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Characterization of the interactions of zinc with known and novel allosteric modulators of glycine receptor function

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Characterization of the interactions of zinc with known and novel allosteric modulators of glycine receptor function

by

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Dedication

This dissertation is dedicated to my mom and dad, whose love and guidance have led me to be the person I am today, and to my wonderful wife, Natasha, without whom I would have never reached this accomplishment.

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My life during the time I was in graduate school has not always been easy, and there are several individuals without whom I would have never accomplished this goal. First, I want to thank my wonderful wife Natasha. You are hands down my number one source of motivation, both in work and in life. I can unequivocally say that you changed the course of my life and that I would not be where I am today without you. You were my inspiration for returning to the graduate program after I had decided to return home and left with my master's degree. Because of this, I have now landed my dream job, studying Alzheimer's disease, something I have been pursuing since I first became interested in science as a child. Your continuous support and advice over the past couple of years has been instrumental in my progress, and for that I cannot thank you enough.

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as two of my favorite courses in all of my academic career. I always enjoyed going to Ion Channel Journal Club, and will miss it greatly when I am gone. Dr. Harris, you too have always been very supportive of my work and career and I believe your help was instrumental in landing my postdoctoral position with Dr. Bess Frost. Dr. Stavchansky, I am in your debt for going out of your way to serve on my committee and working with me in the scheduling of my dissertation defense. I think you have provided an excellent point of view in the examination of my work, and that my dissertation is stronger because of it.

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Characterization of the interactions of zinc with known and novel allosteric modulators of glycine receptor function

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The University of Texas at Austin, 2016

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The glycine receptor is a member of the Cys-loop receptor superfamily of ligand-gated ion channels and is implicated as a possible therapeutic target for the treatment of diseases such as alcoholism and inflammatory pain. In humans, four glycine receptor subtypes (α1, α2, α3, and β) co-assemble to form pentameric channel proteins as either α homomers or $\alpha\beta$ heteromers. To date, few agents have been identified that can selectively modulate the glycine receptor, especially those possessing subtype specificity. We used a cell-based method of phage display panning, coupled with two-electrode voltage-clamp electrophysiology in *Xenopus laevis* oocytes, to identify novel heptapeptide modulators of the α1β glycine receptor. Peptides were identified that act with selectivity on α1β and α3β, compared to α2β, glycine receptors. In addition, peptide activity at the glycine receptor decreased when zinc was chelated by tricine, similar to previous observations of a decrease in ethanol's enhancing actions at the receptor in the absence of zinc. Zinc is an allosteric modulator of glycine receptor function, enhancing the effects of glycine at nanomolar to low micromolar concentrations, and inhibiting its effects at higher concentrations. As zinc is present physiologically at various concentrations within this range, it is capable of influencing glycine receptor function, including modulation by other pharmacological agents;

however, the magnitude of this effect and its possible relevance are not known. I therefore investigated the utility of previously-described "zinc-enhancement insensitive" α1 glycine receptor mutants D80A, D80G, and W170S to probe for interactions between zinc and other allosteric modulators at the glycine receptor. Interestingly, I found that only the W170S mutation conferred complete abolishment of zinc enhancement across a variety of agonist and zinc concentrations. Using α1 W170S receptors, I established that in addition to ethanol, zinc also interacts with inhaled drugs of abuse, but not volatile anesthetics, to synergistically enhance channel function. Additionally, I determined that this interaction is abolished at higher zinc concentrations, when receptor-enhancing bindings sites are saturated, suggesting a mechanism by which modulators such as ethanol and inhalants are capable of increasing receptor affinity for zinc in addition to enhancing channel function on their own.

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List of Abbreviations

5-HT$_3$R	serotonin type 3 receptor
ANOVA	analysis of variance
CBD	cannabidiol
CSF	cerebrospinal fluid
CNS	central nervous system
DH-CBD	dehydroxyl-cannabidiol
EC	effective concentration
ECD	extracellular domain
EtOH	ethanol
GABA$_A$R	γ-aminobutyric acid type A receptor
GlyR	glycine receptor
HEK	human embryonic kidney
ICP-MS	inductively-coupled plasma mass spectrometry
MAC	minimum alveolar concentration
MTSEA	2-aminoethyl-methanesulfonate
nAc	nucleus accumbens
nAChR	nicotinic acetylcholine receptor
PGE$_2$	prostaglandin E_2
PKA	protein kinase A
PMTS	propyl-methanethiosulfonate
SEM	standard error mean
Tau	taurine
TCE	1,1,1-trichloroethane

TCY	trichloroethylene
TM	transmembrane
Tol	toluene
Tri	tricine
VTA	ventral tegmental area
WT	wildtype

Amino Acid Residue Abbreviations and Naming Conventions

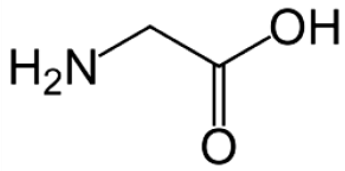
The amino acid residues discussed in this dissertation will often be abbreviated using their single letter code (listed below). Point mutations made within a protein are described using the following nomenclature: original residue-residue position-new residue. For example, the mutation of a tryptophan at position 170 within a protein to serine would be referred to as W170S.

Single-letter amino acid abbreviations

A	Alanine	M	Methionine
C	Cysteine	N	Asparagine
D	Aspartate	P	Proline
E	Glutamate	Q	Glutamine
F	Phenylalanine	R	Arginine
G	Glycine	S	Serine
H	Histidine	T	Threonine
I	Isoleucine	V	Valine
K	Lysine	W	Tryptophan
L	Leucine	Y	Tyrosine

Structures of Glycine Receptor Ligands Relevant to this Dissertation

AGONISTS



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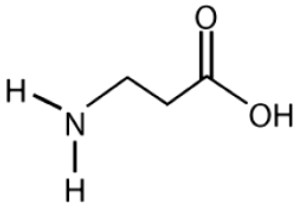
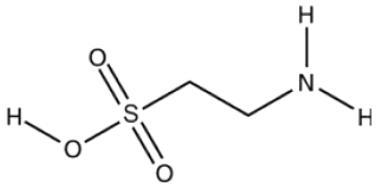


β Alanine



Taurine

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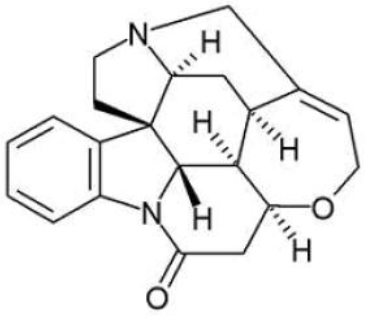

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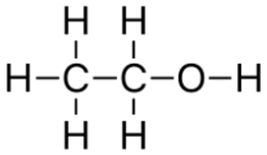
MODULATORS



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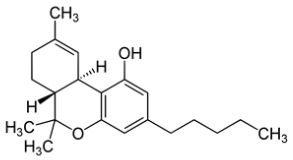
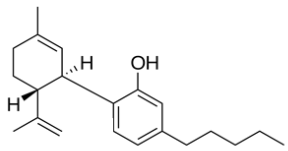
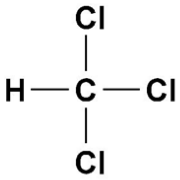


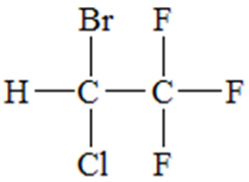
Δ9-Tetrahydrocannabinol



Dehydroxyl-cannabidiol



Chloroform



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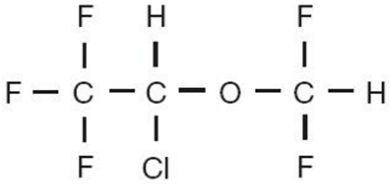

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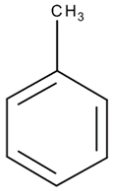
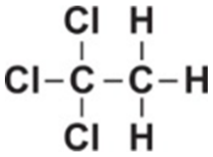
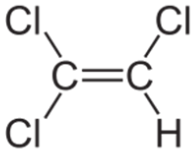


Toluene



1,1,1-trichloroethane
(TCE)



Trichloroethylene
(TCY)

Chapter 1: Introduction

The human nervous system is responsible for integrating and disseminating information throughout the body, controlling processes ranging from sensory perception and motor control to memory, emotion, and cognition. This is achieved by means of a vast network of billions of interconnected electrically-excitable cells known as neurons. Neurons are capable of processing and transmitting information with one another at synaptic junctions by both electrical and chemical means. Neuronal membranes contain a variety of ion channels that allow for the regulated movement of ions in and out of the cell. This permits the propagation of electrical signals, known as action potentials, towards presynaptic terminals where they trigger the release of chemical signaling molecules, or neurotransmitters, into the synaptic cleft. Neurotransmitters diffuse across synapses and convey information to adjacent neurons via the activation of postsynaptic neurotransmitter receptors. Ionotropic neurotransmitter receptors, or ligand-gated ion channels, are key mediators of rapid neurotransmission. These receptors are directly activated by the binding of their cognate neurotransmitter ligands, resulting in the opening of ion conducting pores. This allows for the rapid conversion of neurotransmitter-mediated signaling into changes in membrane potential that affect subsequent neuronal activity.

The Cys-loop receptor superfamily constitutes a major class of ligand-gated ion channels involved in rapid excitatory and inhibitory neurotransmission throughout the nervous system. The prototypical member of this family, being the first discovered and most studied, is the excitatory cation-selective nicotinic acetylcholine receptor (nAChR). Other members include the excitatory, cation-selective serotonin type 3 receptor (5-HT_3R), and the inhibitory, anion-selective channels, the γ-aminobutyric acid type A

receptor ($GABA_AR$) and the glycine receptor (GlyR) (Thompson et al., 2010). These receptors are the pharmacological targets for a wide variety of prescribed medications: The nAChR, the *in vivo* target of nicotine, is targeted for smoking cessation and for the treatment of cognitive decline in dementias such as Alzheimer's disease (Dineley et al., 2015); Antagonists of 5-HT_3Rs are powerful anti-emetics often used to treat radiation and chemotherapy-induced nausea and vomiting (Thompson and Lummis, 2007); and $GABA_A$Rs are commonly targeted for the treatment of epilepsy, insomnia, and anxiety (Tan et al., 2011). The GlyR, apart from perhaps being involved in the effects of volatile anesthetics, is not targeted by any currently-prescribed medications. However, work over the past decade suggests that agents capable of enhancing GlyR function may be able to treat chronic inflammatory pain and substance abuse disorders, such as alcoholism (Li et al., 2012; Molander et al., 2005; Molander and Soderpalma, 2005a; Xiong et al., 2011, 2012). This has led to renewed interest in GlyR pharmacology and a push to develop and characterize GlyR modulators that could serve as potential therapeutic agents.

1.1- The Glycine Receptor

1.1.1- Basic Structure and Function

The GlyR, like all Cys-loop receptors, is composed of five subunits that form a pentamer around a central ion-conducting pore. Each subunit consists of a large extracellular N-terminal domain (ECD), four transmembrane domains (TM1-4), and a large, poorly conserved intracellular domain between TM3-4 (Lynch, 2004). An illustration of these structural components can be seen in **Fig. 1.1**. The TM2 region of each subunit contributes to forming the ion-conducting pore, while the ECD contains both the neurotransmitter binding site and the superfamily's eponymous Cys-loop,

formed by a conserved region of 13 amino acids flanked by cysteine residues that form a disulfide bond (Lynch 2004; Thompson et al., 2010). Like $GABA_A$Rs, the GlyR pore is anion permeable, allowing for the conductance of Cl^- ions across neuronal membranes *in vivo*. Since the resting membrane potential of most neurons is generally less negative than, or close to, the Cl^- reversal potential, GlyR channel opening will result in Cl^- influx and prevention of action potential firing, leading to its classification as primarily an inhibitory neurotransmitter receptor.

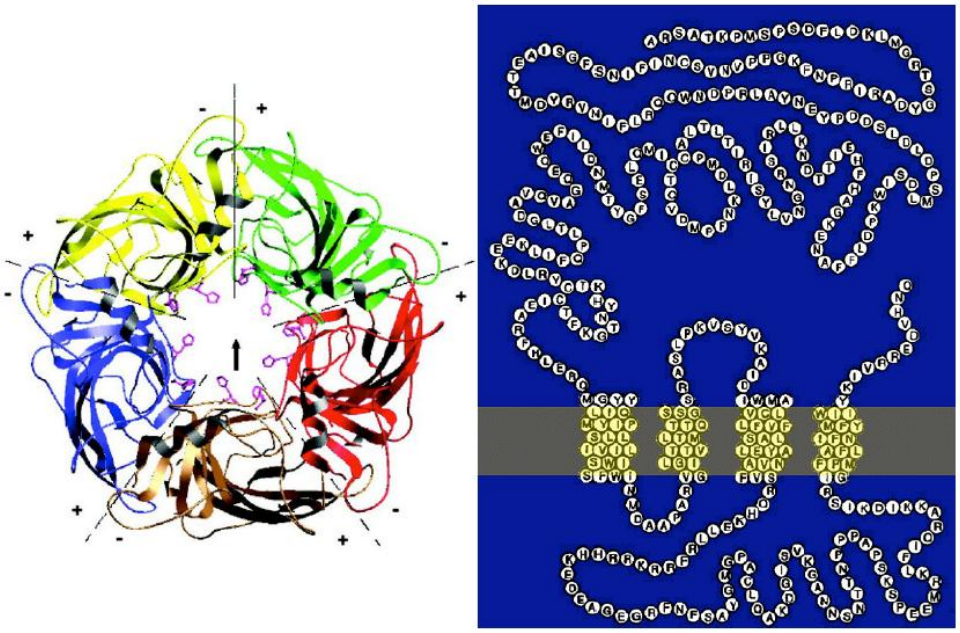


Figure 1.1: Structural components of the glycine receptor

Left: Model of the pentameric structure of the α1 GlyR viewed top down from the extracellular side of the membrane. Each color denotes a separate subunit. Adapted from *Lynch JW. 2004. Molecular structure and function of the glycine receptor chloride channel. Physiol Rev 84, 1051-1095.*

Right: Cartoon representation of a single subunit of the α1 GlyR. Each amino acid is denoted by a circle and the amino acid's single letter code. The yellow band represents the plasma membrane with the residues of each transmembrane domain clustered within. Adapted from *Rajendra et al. 1997. The glycine receptor. Pharmacol Ther 73, 121-146.*

To date, four GlyR subunits, three α and one β, have been identified in humans (Lynch, 2004). A fourth α subunit is present in rodents, but is a pseudogene in humans (Lynch, 2004). The α-subunits display high levels of homology, sharing 80-90% primary amino acid sequence identity, while the β subunit displays ~47% similarity with the α1-subunit (Lynch, 2004). Subunits combine to form functional α homomeric or αβ heteromeric channels. The β subunit, although widely expressed throughout the nervous system, is unable to form functional homomeric receptors. The β subunit is responsible for postsynaptic localization of receptors via interactions with the postsynaptic scaffolding protein gephyrin; heteromeric receptors are generally considered synaptic while homomeric receptors are considered extra-synaptic (Kirsch and Betz, 1995; Meyer et al., 1995). The stoichiometry of heteromeric receptors is still somewhat unclear, with recent reports of both 2α:3β and 3α:2β (Durisic et al., 2012; Yang et al., 2012).

As the receptor's name implies, the amino acid glycine serves as the primary ligand responsible for GlyR activation. In the absence of ligand, channels exist in a closed state with little to no spontaneous activity. Ligand binding occurs at the interface of the ECDs of adjacent subunits, allowing for up to 5 possible ligand-binding sites per receptor (Brejc et al., 2002). Agonist binding at a single site results in the simultaneous conformational change of all subunits to the activated state (Corringer et al., 2000), and single channel conductance is the same at all concentrations of glycine tested (Lape et al., 2008). Maximal receptor activation occurs when three or more glycine molecules are bound, since the percent time the channel spends in the open state increases with agonist concentration (Lewis et al., 2003; Beato et al., 2004). In addition to glycine, other endogenous amino acids such as taurine and β-alanine are also capable of activating the receptor. These agonists have lower efficacies than glycine, meaning their binding leads to a decreased probability of opening the channel compared to the binding of glycine,

lending to their classification as partial GlyR agonists (Lynch et al., 1997). Studies of taurine levels in certain brain regions have led to speculation that taurine, and not glycine, may act as the primary ligand of GlyRs found in these areas (Mori et al., 2002; Ericson et al., 2006).

1.1.2 - Allosteric Modulation

In addition to activation by orthosteric ligands, the GlyR is subject to allosteric modulation by a wide variety of compounds including alcohols, inhalants, anesthetics, cannabinoids, divalent cations such as zinc, and others (Beckstead et al., 2000; Cheng and Kendig, 2002; Downie et al., 1996; Harvey et al., 1999; Laube et al., 1995; Molander et al., 2007, 2005; Yamashita et al., 2001; Yevenes and Zeilhofer, 2011). Most of these compounds, particularly the drugs of abuse, are positive allosteric modulators that enhance GlyR function, resulting in a left-shift of glycine concentration response curves (Yevenes and Zeilhofer, 2011). These compounds bind the receptor at sites distinct from the orthosteric ligand-binding site and enhance ligand-activated currents, but have no effect when applied to receptors in the absence of ligand. Allosteric modulators could conceivably enhance receptor function via four possible mechanisms: 1) increasing the affinity of the receptor for its orthosteric ligand; 2) increasing the orthosteric ligand's ability to open the channel once bound, i.e., its efficacy; 3) increasing the channel's conductance; or 4) by decreasing the rate of orthosteric- ligand-induced receptor desensitization. Zinc and ethanol fail to enhance currents generated by saturating concentrations of glycine, suggesting these allosteric modulators increase agonist affinity, not efficacy or conductance. Additionally, zinc and ethanol enhance rather than antagonize the rates of glycine-mediated desensitization. Analysis of single channel recordings of both zinc and ethanol's effects on the α1 GlyR shows that these modulators

increase channel burst durations but do not significantly affect the probability of within-burst channel opening (Laube et al., 2000; Welsh et al., 2009). These data are in accordance with a mechanism of modulator action that involves increasing glycine's affinity by decreasing its rate of unbinding from the receptor. However, currents generated by saturating concentrations of the partial agonist taurine are still sensitive to enhancement by both compounds, suggesting they do increase taurine's efficacy and that mechanisms of allosteric modulation may depend on the agonist present (Kirson et al., 2012, 2013).

Allosteric modulators of the glycine receptor have a variety of putative binding sites. The key residues involved in the binding of the compounds discussed here are illustrated in **Fig. 1.2**. Drugs of abuse such as ethanol, inhalants, volatile anesthetics, and cannabinoids are all thought to have overlapping binding sites within an intersubunit water filled cavity involving TM2 and TM3. A molecular dynamics simulation of ethanol's interactions with residues in this pocket can be seen in **Fig. 1.3**. This concept was first established by Mihic et al. (1997) who utilized a variety of chimeric receptors created from α1 GlyR and $GABA_A$-ρ1 subunits, which have differential sensitivities to anesthetics and alcohol, to identify a 45 amino acid region between TM2 and TM3 of the α1 GlyR that was necessary and sufficient for conferral of receptor enhancement by enflurane and ethanol. Subsequent site-directed mutagenesis of individual residues in this region identified residues S267 in TM2 and A288 in TM3 as critical determinants of modulator function, suggesting a role in modulator binding (Mihic et al., 1997; Ye et al., 1998; Yamakura et al., 1999). This notion was further supported by covalent modifications of S267C and A288C mutant receptors with propyl-methanethiosulfonate (PMTS), a thiol reagent that forms a disulfide bond with the mutated cysteines to mimic modulator binding. Modification of S267C and A288C residues with PMTS resulted in a

permanent enhancement of GlyR function that was not further potentiated by alcohol or anesthetics, consistent with molecular occupation of this site conferring GlyR enhancement by these compounds (Mascia et al., 2000). More recently, the existence of this binding pocket has been confirmed by the determination of co-crystal structures of homologous prokaryotic ligand-gated ion channels bound by a variety of modulators (Nury et al., 2011; Sauguet et al., 2013; Spurny et al., 2013). Site directed mutagenesis and NMR studies have also shown that the cannabinoids Δ^9-tetrahydrocannabinol and cannabidiol (CBD) enhance GlyR function through interaction in this region at S296 (Xiong et al., 2011, 2012).

Zinc modulation of the GlyR is unique in that zinc appears to have at least two distinct binding sites that confer opposite effects. Zinc is a biphasic modulator of GlyR function, enhancing GlyR currents below 10 μM while causing inhibition at higher concentrations (Laube et al., 1995). As mutations that affect zinc-mediated GlyR enhancement do not affect inhibition, and vice versa, these sites are believed to be completely separate (Miller et al., 2005; Nevin et al., 2003), suggesting the presence of both a high-affinity GlyR-enhancing site and a low-affinity GlyR-inhibitory site. The zinc inhibitory binding site is thought to be located at an inter-subunit interface of the extracellular domain where zinc is ligated by H107 of one subunit and H109 of the other (Nevin et al., 2003). These residues were initially implicated in a study that found that histidine-specific modifying agents were capable of reducing zinc-mediated GlyR inhibition (Nevin et al., 2003). Subsequent site-directed mutagenesis of extracellular histidine residues led to the identification of H107 and H109 specifically. Intersubunit binding of zinc at this location was supported by molecular modeling and the fact that the reduced zinc inhibition seen in homomeric α1 H107A or H109A GlyRs was rescued

when these subunits were co-expressed, consisting with an intersubunit coordination of zinc (Nevin et al., 2003).

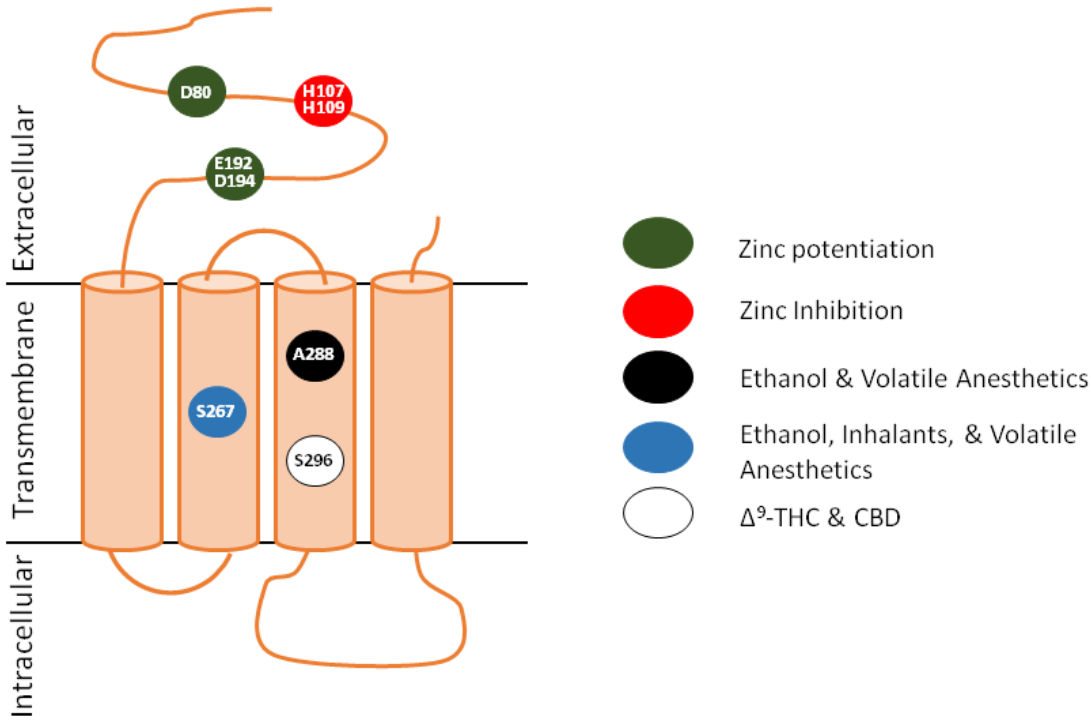


Figure 1.2: Key residues thought to be involved in allosteric modulation of the glycine receptor by zinc and drugs of abuse.

Zinc has both GlyR-enhancing and inhibitory effects mediated by at least two distinct sites in the extracellular domain. Drugs of abuse enhance GlyR function by interaction with a water filled cavity formed by TM2 and TM3. Adapted from *Yevenes GE and Zeilhofer HU. 2011. Allosteric modulation of glycine receptors. British J Pharmacol. 164, 224-236.*

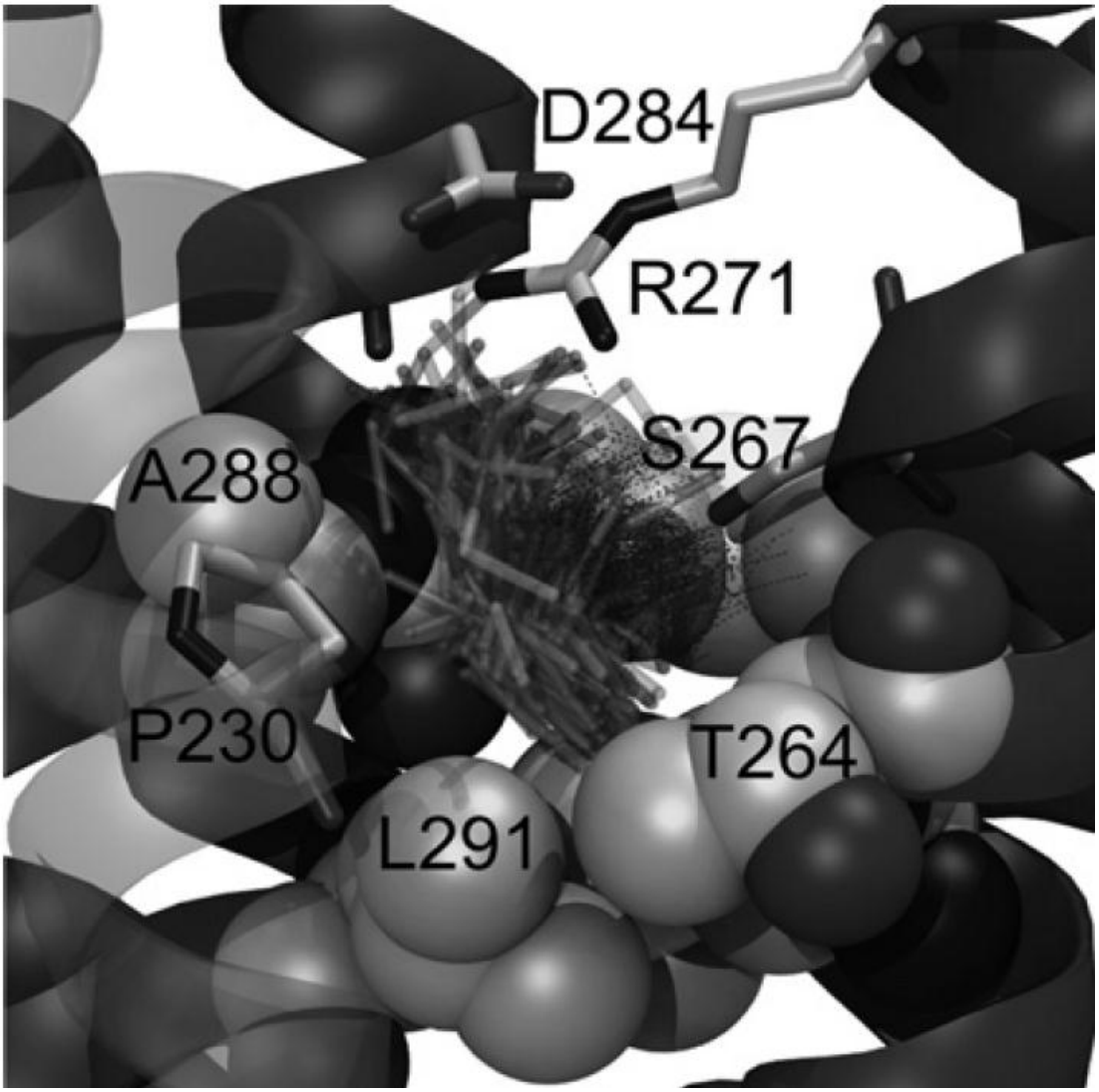


Figure 1.3: Molecular dynamic simulation of ethanol binding at the α1 glycine receptor

A 229 ns simulation of ethanol binding to a homology model of the α1 GlyR based on the crystal structure of the *Gloeobacter violaceus* pentameric ligand-gated ion channel. Ethanol, depicted as transparent sticks, stably occupied the putative alcohol-binding site between transmembrane domains 2 and 3 via hydrogen bonds with S296 (dashed lines) and hydrophobic packing with A288, M287 (not shown), and L291. Adapted from *Murail et al., 2011. Microsecond simulations indicate that ethanol binds between subunits and could stabilize an open-state model of a glycine receptor. Biophys J 100, 1642-1645*

The high affinity GlyR-enhancing zinc binding site is less defined. The first proposed binding site involved ECD residue D80, as neutral substitutions of this position abolished, or significantly inhibited, zinc-mediated enhancement of glycine-activated currents (Laube et al., 2000; Hirzel et al., 2006). However, one report found that zinc potentiation was retained in D80A GlyRs activated by taurine (Lynch et al., 1998). Further, this same study found that enhancing concentrations of zinc cause a similar increase in the apparent affinity of D80A mutant and wildtype receptors to both glycine and taurine in [^{3}H] strychnine binding assays, suggesting zinc retains the ability to bind to and affect the channel. Together, these data suggest that neutral substitutions of D80 produce an allosteric effect on zinc modulation, as opposed to abolishment of zinc binding. The other proposed GlyR-enhancing zinc binding site involves extracellular residues E192 and D194 (Miller et al., 2005). Neutral substitutions at either position completely abolish zinc-enhancement of both glycine and taurine-activated receptors. Additionally, covalent modifications of cysteine mutations incorporated at these positions with 2-aminoethyl-methanesulfonate (MTSEA), a thiol reactive reagent similar to PMTS, were consistent with their role in mediating the binding of enhancing concentrations of zinc. Specifically, the labeling of E192C or D194C with MTSEA produced a potentiation of GlyR currents that could not be further enhanced by the application of zinc (Miller et al., 2005). However, zinc-inhibition was retained in labeled mutant receptors, providing further evidence for distinct GlyR enhancing and inhibitory zinc binding sites (Miller et al., 2005).

1.1.3 - The Need to Account for the Effects of Zinc in Studies of Glycine Receptor Function

Rapidly exchangeable zinc (referred to as “free” zinc) is ubiquitous in the central nervous system and found tonically in cerebrospinal fluid (CSF) at nanomolar

concentrations known to be sufficient to enhance GlyR function (Frederickson et al., 2006b). Zinc can also be released synaptically, resulting in higher local concentrations reported to range from 1-100 μM (Frederickson et al., 2006a; Qian and Noebels, 2005; Vogt et al., 2000; Zhang et al., 2016). Evidence of zinc's release at glycinergic synapses was recently reported by Zhang et al. (2016) who found that local zinc concentrations can reach up to 1 μM after presynaptic stimulation of primary cultured rat embryonic glycinergic neurons. Additionally, zinc modulation appears to be important for proper GlyR function as mutations that result in the abolishment of zinc-enhancement have been shown to cause human hyperkplexia and hyperkplexia-like phenotypes in rodents (Hirzel et al., 2006; Zhou et al., 2013).

In addition to its presence *in vivo*, zinc is a common contaminant that can be found in labware and reagents at concentrations capable of affecting GlyR function (Cornelison and Mihic, 2014; Kay, 2004). We previously determined that our electrophysiological buffers contain approximately 45 nM zinc (Cornelison and Mihic, 2014). Other studies have reported approximately 200-800 nM contaminating zinc in electrophysiological solutions (Thio and Zhang, 2006; Wilkins and Smart, 2002; Zheng et al., 1998). All of these concentrations are within the GlyR-enhancing range suggesting that, without accounting for the effect of contaminating zinc, one cannot assume that responses elicited by glycine alone are not necessarily also partially due to some level of allosteric modulation by zinc. To complicate matters further, recent reports have shown that the degree of ethanol enhancement of GlyR function is dependent on the concentration of zinc present. Specifically, chelation of contaminating zinc significantly decreased ethanol enhancement of GlyR function while the addition 50-500 nM zinc significantly increased ethanol enhancement of GlyR function (McCracken et al., 2010;

2013a,b). Since contaminating zinc is seldom accounted for in studies of GlyR function, the extent of zinc-dependence in the action other GlyR modulators is unknown.

1.2- The Glycine Receptor as Therapeutic Target

1.2.1 – Alcoholism

Due to its ease of access, and the relative lack of social stigma attached to its use, alcohol has become one of the most widely used drugs of abuse. The 2014 *National Survey on Drug Use and Health* conducted by the Substance Abuse and Mental Health Services Administration reported that 16.3 million, or 6.8%, of adults over the age of 18 in the U.S. suffered from alcohol use disorder. Further, nearly 679,000, or 2.7%, of adolescents were also reported suffer from alcohol use disorder. Between 2006 and 2010, the U.S. saw an average of 87,790 alcohol-associated deaths a year with nearly 1 in 10 deaths of working-age adults (20-64 years of age) attributed to excessive alcohol consumption (Stahre et al., 2014). Additionally, excessive alcohol consumption was estimated to cost the U.S. $249 billion in 2010 (Sacks et al., 2015). Ethanol, the active ingredient in alcoholic beverages, is a central nervous system (CNS) depressant that is believed to exert its effects through a large number of molecular targets (Harris et al., 2008). The non-specific nature of ethanol's actions has made it difficult to determine viable therapeutic targets for pharmacological intervention.

Like other drugs of abuse, ethanol increases dopamine signaling from the ventral tegmental area (VTA) to the nucleus accumbens (nAc), an effect thought to be critical in the rewarding properties of drugs of abuse (Gonzales et al., 2004). Multiple lines of evidence now suggest that ethanol enhancement of GlyRs located on accumbal GABAergic neurons disinhibit dopaminergic neurons, resulting in ethanol-mediated

dopamine release. Activation of presynaptic GlyRs on GABAergic interneurons in the VTA has been shown to facilitate the firing of dopaminergic cells (Ye et al., 2004). Additionally, infusion of glycine into the nAc increased dopamine concentration, similar to alcohol, and alcohol-induced dopamine release was blocked by the GlyR-specific competitive antagonist strychnine (Molander and Soderpalm 2005a,b; Molander et al., 2005). Further, infusion of glycine into the nAc or microinjection of glycine into the VTA have both been shown to selectively decrease ethanol consumption and preference in rodents (Li et al., 2012; Molander and Soderpalm, 2005a,b; Molander et al., 2005). Moreover, the glycine reuptake inhibitor Org 25935, which increases GlyR function by preventing reuptake of extracellular glycine, increases dopamine levels in the nAc, with decreased alcohol consumption and preference seen in rodents (Lidö et al., 2009, 2010; Molander et al., 2007). These studies suggest that GlyR-enhancing agents may be able to satiate cravings for alcohol via increased dopamine release in the absence of alcohol consumption. A recent clinical trial found no benefit of Org 25935 over placebo in the preventions of relapse in alcohol dependent patients (de Bejczy et al., 2014). However, due to a number of limitations within the trial itself, research is expected to continue on this molecule and other GlyR-enhancing agents.

1.2.2 – Chronic Inflammatory Pain

The α3 GlyR is abundantly expressed in lamina II neurons of the spinal dorsal horn, where the majority of nociceptive afferents terminate (Harvey et al., 2004). Increased function of these receptors is thought to gate the relay of nociceptive signaling into higher brain regions (Ahmadi et al., 2002; Foster et al., 20015; Harvey et al., 2004, 2009). Prostaglandins have long been recognized as important mediators of inflammatory pain (O'Banion 1999). Prostaglandin E_2 (PGE_2) has been shown to

selectively mediate inflammatory pain via protein kinase A (PKA)-dependent inhibition of α3 GlyR function (Ahmadi et al., 2002; Harvey et al., 2004, 2009; Hosl et al., 2006). Specifically, PGE_2 activation of PKA results in phosphorylation of GlyR residues in the intracellular loop that have been shown to induce both receptor internalization and conformational changes that decrease receptor sensitivity to agonist (Han et al., 2013; Huang et al., 2007; Velázquez-Flores and Saleda 2011).

Recently, the cannabinoid dehydroxyl-CBD (DH-CBD) was shown to rescue PGE_2 inhibition of α3 GlyR function (Xiong et al., 2012). Additionally, cannabinoid-induced analgesia is thought to be primarily mediated through positive allosteric modulation of GlyRs (Xiong et al., 2011, 2012). Cannabinoid-induced analgesia in rodent models of thermal and mechanical pain was abolished in α3 GlyR knockout mice but unchanged in cannabinoid receptor type 1 and 2 knockout mice (Xiong et al., 2011). There was also a strong positive correlation between the analgesic potency of 11 different cannabinoids and their abilities to potentiate the α3 GlyR, but there was no correlation seen between their analgesic effects and their affinities for cannabinoid receptors (Xiong et al., 2011). Further, it was shown that a DH-CBD analogue that antagonizes DH-CBD enhancement of GlyR function also antagonizes DH-CBD-induced analgesia in rodent models of inflammatory pain (Xoing et al., 2012). Interestingly, there was no loss in the analgesic effect of DH-CBD with repeated administrations over multiple days, suggesting a lack of tolerance development for this molecule. This is especially promising as tolerance is a large problem with currently prescribed medications for chronic pain. Taken together, these data suggest agents capable of enhancing α3 GlyR function could serve as valuable novel therapeutics in the treatment of chronic inflammatory pain.

1.3 - Phage Display

Historically, the discovery of novel drugs for the treatment of disease has relied upon serendipity or the isolation of active compounds in known natural remedies and their subsequent chemical modification. However, the development of high-throughput drug screening platforms and an ever-increasing body of knowledge of the molecular basis of disease has allowed for a more rational approach to drug discovery (Drews, 2000). Phage display has long been utilized to identify peptides, antibodies, or other proteins capable of binding to specific molecular targets (Molek et al., 2011; Nixon et al., 2014). Now considered a crucial drug discovery platform, phage display has contributed to the development of a variety of approved medications including the popular arthritis treatment Humira, the protective anthrax antibody ABthrax, the lupus medication Benlysta, and others (Nixon et al., 2014).

Phage display involves the production of large libraries of bacteriophage that have been genetically modified to display peptides as fusion proteins with various phage coat proteins. A variety of phage display platforms have been developed. The most common systems employ fusions or peptides or antibodies to minor coat proteins of the Ff family of filamentous phage (M13, Fd, f1) (Buckler et al., 2012; Nixon et al., 2014). Phage display libraries of large molecular diversity have been generated with the ability to display up to 10^{11} distinct peptide sequences (Sidhu et al., 2000). Libraries are 'panned' against a protein target, generally immobilized to the surface of a plate, and phage that bind specifically to the target can be isolated and amplified by outgrowth in bacteria. The direct physical linkage of the displayed protein to the gene that encodes it facilitates easy identification of the peptides responsible for the binding of phage to molecular targets. Over several rounds of selection and outgrowth amplification, target-specific peptides

can be readily selected from a phage library. An illustration of a basic phage display procedure can be seen in **Fig. 1.4**.

The affinity of selected peptides or proteins for their targets depends largely upon the type of library and methods used. The valence of displayed peptides has a strong effect on subsequent peptide affinity and depends on what phage coat protein the peptide is fused to, ranging from five to thousands of copies per phage using standard platforms (Molek et al., 2011). Polyvalent display leads to an avidity effect where the apparent affinity of the phage-peptide complex, containing multiple copies of the peptide that are all capable of binding the target, is greater than that of a single peptide alone (Lowman, 1997). This can be overcome through the utilization of a phagemid-display system. This method utilizes the transformation of phagemids encoding the displayed fusion proteins into bacteria. Subsequent infection of bacteria with "helper-phage" provides the remainder of genes necessary for mature phage production. The competition of phagemid encoded peptide-displaying coat proteins with wildtype proteins results in a decreased valency of the displayed peptide and can be optimized to result in monovalent display (Qi et al., 2012). Other factors that influence the affinity of identified peptides for target include the concentration of target used in panning, the amount of time allowed for phage binding, and the stringency of subsequent wash steps. Generally, naïve libraries result in the identification of peptides or proteins that can bind with mid nM - high μM affinities, although sub-nM affinity has been achieved (Buckler et al., 2012; Lowman, 1997; Molek et al., 2011; Nixon et al., 2014; Qi et al., 2012). In order to obtain binding affinities appropriate for therapeutic use (low nM), researchers typically employ an iterative affinity maturation process in which the sequences of identified peptides are used to design new libraries for subsequent series of panning (Nixon et al., 2014; Thie et al., 2009).

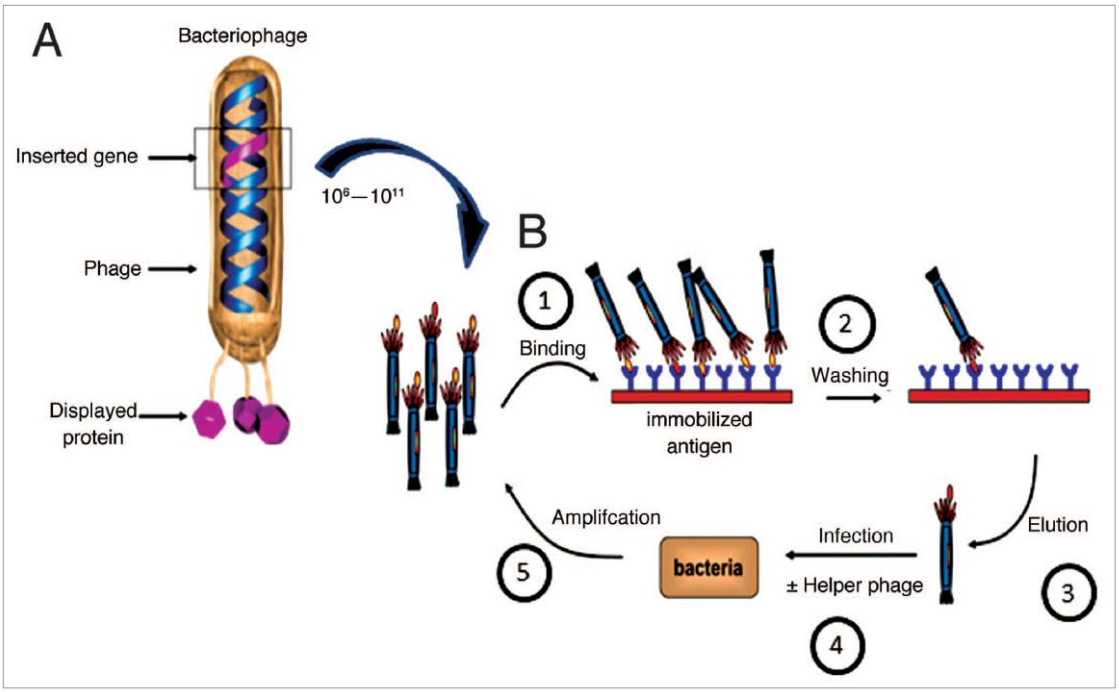


Figure 1.4: Illustration of a basic phage display procedure

A) Cartoon model depicting a basic bacteriophage used in phage display. The gene encoding a displayed peptide (pink) is cloned into the pIII minor coat protein. B) Overview of a basic phage display panning procedure: 1) A phage library containing up to 10^{11} unique clones is incubated with target antigen. 2) Unbound phage are removed by a series of wash steps. 3) Bound phage are eluted, often through a reduction in the pH. 4) Eluted phage are used to infect bacteria. 5) Phage replicate in bacteria, amplifying the number of phage that display peptides capable of binding to the target antigen. These phage can then be isolated and used in subsequent rounds of panning to increase the specificity and affinity of identified peptides. Standard phage display procedures employ a series of 3-5 rounds of panning prior to characterization of selected peptides. The sequence of selected peptides are often used to design new libraries for subsequent series of panning in order to further increase the affinities of identified peptide for their target. Adapted from *Nixon et al., 2014. Drugs derived from phage display: from candidate identification to clinical practice. mABs 6, 73-85.*

Standard phage display procedures call for the purification and fixation of target proteins to microtiter plates, complicating the selection of peptides capable of affecting the function of integral membrane proteins (Molek et al., 2011). This is partially due to the fact that the transmembrane regions of these proteins are extremely hydrophobic and difficult to purify. This can be overcome through the targeting of smaller recombinant protein fragments, such as the extracellular domains, that can easily be purified and fixed to a plate (Su et al., 2005; Hetian et al. 2002; Molek et al., 2011). However, multimeric membrane proteins, such as ligand-gated ion channels, require interactions between multiple protein subunits for the maintenance of their proper functional conformation. For this purpose, phage display procedures have been developed in which phage libraries are panned against targets expressed in cultured cells, ensuring their proper functional conformation (Arita et al., 2016; Tipps et al., 2010; Wang et al., 2006; Watters et al., 1997). However, in order to avoid the selection of phage capable of binding non-specifically to the cells themselves, a negative selection step is often required (Molek et al., 2011). This involves a process referred to as 'subtractive panning,' in which phage libraries are washed over cells that do not express the target of interest prior to positive selection against target-expressing cells. This allows for the depletion of phage clones from the library that would be capable of nonspecifically binding endogenous proteins found on the cells utilized for expression of the target protein. Tipps et al. (2010) reported the first adaptation of a phage display procedure to target a ligand-gated ion channel, specifically the GlyR. This study employed a cell based phage display procedure in which GlyRs were overexpressed in human embryonic kidney (HEK) cells for positive selection while subtractive panning was carried out in un-transfected HEK cells. Using this method, they were able to identifying peptides capable of selectively

modulating GlyR function at low μM concentration while minimally affecting the closely related $GABA_AR$.

Chapter 2: Materials and Methods

Materials and methods common among the studies contained within this dissertation are presented below. Methods that are specific to a particular study can be found within the materials and methods section of the appropriate chapter.

2.1- Buffers and reagents

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) except for isoflurane which was obtained from Marsam Pharmaceutical Inc. (Cherry Hill, NJ), tricaine which was obtained from Western Chemical, Inc. (Ferndale, WA), and synthetic peptides which were obtained from Peptide 2.0 (Chantilly, VA).

All buffers were made in ultrapure diH_2O and pH adjusted with NaOH as necessary. Buffer recipes were as follows:

Modified Barth's Saline (MBS) — 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES, 0.82 mM, $MgSO_4 \cdot 7H_2O$, 0.33 mM $Ca(NO_3)_2$, and 0.91 mM $CaCl_2$, pH 7.5

Isolation Media — 108 mM NaCl, 1 mM EDTA, 2 mM KCl, and 10 mM HEPES, pH 7.5

Collagenase Solution — 83 mM NaCl, 2 mM $MgCl_2$, and 5 mM HEPES, 0.5 mg/ml Sigma Type 1A collagenase, pH 7.5

Incubation Media — MBS + 2 mM sodium pyruvate, 0.5 mM theophylline, 10 U/ml penicillin, 10 mg/l streptomycin, and 50 mg/l gentamicin

2.2- Xenopus laevis ooctye isolation and cDNA injection

Xenopus laevis were obtained from Nasco (Fort Atkinson, WI) and housed at 19°C on a 12 hr light/dark cycle. Oocytes were surgically obtained in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care regulations. Briefly, frogs were anesthetized with tricaine and a small incision was made in the lower abdomen with a no. 10 scalpel. A small portion of the ovaries was removed with forceps and stored in incubation media. Incisions were then closed with sutures and frogs were allowed to recover in isolation for a minimum of three hours before being returned to their home tanks. Frogs were allowed to fully recover in their home tanks for at least 30 days prior to subsequent surgeries. After three surgeries, frogs were euthanized by an overdose of tricaine.

Harvested oocytes were placed in hypertonic isolation media in order to slightly shrink the oocyte for easier removal of protective membranes. Forceps were used to manually remove the thecal and epithelial layers from stage V and VI oocytes before removal of the follicular layer by a 10 min incubation in 0.5 mg/ml type 1A collagenase. Isolated oocytes were then washed and stored in incubation media up to 24 hr prior to cDNA injection.

A 32.2 nl sample of 50 ng/μl of the appropriate GlyR cDNA, contained within a modified pBK-cytomegalovirus vector (Mihic et al., 1997), was injected into the animal pole of each oocyte using a micropipette (10-15 μm tip size) attached to an electronically activated microdispenser. When expression of heteromeric receptors was desired, cDNA was injected at a 1:20 α:β ratio (50 ng/μL total). Oocytes were stored individually at room temperature in 96-well plates containing incubation media sterilized by passage

through a 0.22 μM filter. Oocytes expressed GlyRs within ~24 hr and all electrophysiological recordings were made within 5 days of oocyte isolation.

2.3- Two-electrode voltage-clamp electrophysiology

Oocytes were placed in a 100 μl bath with the animal poles facing upwards and impaled with two high-resistance (0.5-10 MΩ) glass electrodes filled with 3 M KCl. Oocytes were perfused with MBS at a rate of 2 ml/min through 18-gauge polyethylene tubing using a Masterflex peristaltic pump (Cole Parmer Instrument Co., Vernon Hills, IL) and voltage-clamped at -70 mV using an OC-725C oocyte clamp (Warner Instruments, Hamden, CT). One electrode is used to continually measure the voltage of the cell while the other electrode injects current in order to maintain the desired potential. When GlyRs open, chloride ion flux is detected by the voltage electrode, resulting in an injection of current in order to maintain the desired voltage. The magnitude of the injected current is equal and opposite to the flow of ions through the channel, allowing for accurate quantification of GlyR activity. Currents were measured at a rate of 1 kHz using a Powerlab 4/30 digitizer with LabChart version 7 software (ADInstruments, Bella Vista, NSW, Australia).

Chapter 3: Identification and characterization of peptide modulators of the glycine receptor[1]

3.1- Introduction

The Cys-loop receptor superfamily constitutes a major class of ligand-gated ion channels involved in fast inhibitory and excitatory neurotransmission throughout the central nervous system and periphery. Due to their involvement in a variety of central nervous system disorders, several members of this receptor superfamily serve as targets both for compounds in clinical use, as well as investigational agents (Dineley et al., 2015; Nys et al., 2013). In particular, the GlyR has been identified as a potential target for a variety of therapeutic applications, including the treatment of inflammatory pain and alcoholism (Li et al., 2012; Molander et al., 2005; Molander and Soderpalma, 2005a; Xiong et al., 2011, 2012).

Although GlyRs predominate in the brain stem and spinal cord, they are also expressed in higher brain regions such as the nucleus accumbens, frontal cortex, and hippocampus (Jonsson et al., 2012; 2009; Molander and Söderpalm, 2005b). A variety of drugs of abuse, including alcohol, inhalants and volatile anesthetics enhance GlyR function at concentrations that are achieved *in vivo* (Lynch, 2004; Molander et al., 2005; Xiong et al., 2009). Enhanced activation of these channels through positive allosteric modulation effectively treats inflammatory pain in rodents and may aid in the treatment of addictions such as alcoholism, through modulation of dopamine release (Molander et

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[1]Portions of this chapter have previously been published in *European Journal of Pharmacology*. Cornelison, G.L., Pflanz, N.C., Tipps, M.E., Mihic, S.J. 2016. Identification and characterization of heptapeptide modulators of the glycine receptor. *Eur J Pharmacol* 780, 252-259. G.L.C. conducted or supervised all experiments and wrote the manuscript.

al., 2005; Molander and Soderpalm, 2005 a,b; Xiong et al., 2012). However, while α3-containing receptors are thought to predominate in GlyR-mediated analgesia, it is not entirely clear which subtypes are responsible for the behavioral and dopamine-modulating effects of alcohol. While α1– and α2–containing receptors seem to be the most likely in vivo targets of alcohol (Blednov et al., 2015), there would be considerable utility in the development of GlyR modulators with subunit selectivity, to definitively determine the relative contributions of each particular subtype to alcohol's pharmacological effects. Our previous work validated the use of phage display for the discovery of novel peptide modulators of the GlyR (Tipps et al., 2010). In this chapter, we expand on these studies by identifying and characterizing the action of peptides with selectivity for α1β and α3β over α2β– containing GlyRs and identify several possible amino acid consensus sequences within the heptapeptides. Interestingly, the actions of these peptides appear to be zinc-dependent. This zinc dependence was also previously shown to affect alcohol modulation of the GlyR (McCracken et al., 2010), suggesting that the presence of zinc may be necessary for efficient modulation of GlyR activity by allosteric modulators.

3.2- Materials and Methods

3.2.1- Peptide reagents

Peptides were synthesized as HCl salts at 98% purity by Peptide 2.0 Inc. (Chantilly, VA). Peptides were received as a lyophilized powder and suspended at a concentration of 10 mM in ultrapure H_2O, based on the theoretical peptide molecular weight and the dry weight of lyophilized peptide material. Suspended peptides were stored as single-use aliquots at -20°C for up to 4 weeks before use.

3.2.2- Human Embryonic Kidney (HEK) Cell Culture and Expression of Glycine Receptors

HEK 293 cells were obtained from the American Type Culture Collection and grown according to standard procedures (Freshney, 2002). Briefly, cells were cultured at 37°C and 5% CO_2 in Gibco® Dulbecco's modified Eagle's medium with L-glutamine, sodium pyruvate, and 10% fetal bovine serum (Thermo-Fisher Scientific, Waltham, MA). Cells were split every 5 days with Gibco® trypsin-EDTA (Thermo-Fisher Scientific) up to 25 times, after which new aliquots of early-passage cells were started. Cells were transfected with 4 μg of GlyR α1β, α2β, or α3β cDNA (1:20 α:β ratio) in modified pBK-cytomegalovirus vectors (Mihic et al., 1997) using PolyFect reagent (Qiagen, Valencia, CA). All cells were incubated for at least 48 hr before use in panning.

3.2.3- Phage Display

The phage display procedure was modified from the manufacturer's instructions and consisted of two separate panning series, D7.1 and D7.2. Each series was identical except for the subtractive panning step against negative selection cells. This step allows for the depletion of phage capable of binding non-specifically to either endogenous HEK cell membrane proteins or to the overexpressed negative selection receptors, thereby increasing the specificity of obtained peptides. Subtractive panning for series D7.1 consisted of washing the phage library over negative selection HEK 293 cells expressing α2β GlyRs while D7.2 consisted of a pair of washes of library over negative selection HEK 293 cells expressing α2β, followed by HEK 293 cells expressing α3β GlyRs. On panning day 1, plates of positive and negative selection HEK 293 cells were washed three times with 0.01 M phosphate-buffered saline (PBS) containing 8.2 mM NaPO4, 1.5 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl with 1.5% bovine serum albumin (BSA) and 0.1% Tween (PBS/BSA+T). Next, the Ph.D.-7 phage library (New

England Biolabs, Ipswich, MA) was diluted to ~10^{11} plaque forming units (PFU) in 1 ml of PBS/BSA+T, applied to negative selection cells, and rocked gently at room temperature for 30 min. Phage capable of binding non-specifically to endogenous HEK 293 proteins or to the expressed negative selection receptors were thereby removed by this subtractive panning step and the remaining phage in the supernatant were transferred to positive selection HEK 293 cells expressing α1β GlyRs and rocked gently for 60 min. Phage unable to bind to the positive selection target were discarded with the supernatant and phage bound to α1β GlyRs were eluted in 1 mL of phage elution buffer (0.2 M glycine-HCl + 1 mg/ml BSA, pH 2.0) by gentle rocking for 10 min. Eluate was removed and neutralized with 150 μL of 1 M Tris-HCl (pH 9.0).

10 μL of eluate was used to determine phage titer and the remainder was used to infect 20 mL of mid-log phase *Escherichia coli* ER2738 in LB broth. After 4.5 hr of incubation at 37°C, the *E. coli*-phage mixture was pelleted at 10,000 rpm for 10 min at 4°C. The supernatant was transferred to a fresh tube and re-spun. The upper 80% of the supernatant was again transferred to a fresh tube, and one-sixth volume of polyethylene glycol/NaCl (20% w/v polyethylene glycol 8000 and 2.5 M NaCl) was added. Phage were allowed to precipitate overnight at 4°C.

On panning day 2, polyethylene glycol precipitates were spun at 10,000 rpm for 15 min at 4°C. The supernatant was discarded and the precipitate was spun again. Residual supernatant was removed using a pipette and the phage-containing pellet was re-suspended in 1 mL of Tris-buffered saline (50 mM Tris-HCl (pH 7.5) and 150 mM NaCl), transferred to a 1.7 mL microcentrifuge tube and spun at 10,000 rpm for 5 min at 4°C. In a fresh microcentrifuge tube, the suspended phage were re-precipitated with polyethylene glycol/NaCl on ice for 60 min. The precipitate was then spun again at 10,000 rpm for 10 min at 4°C and the supernatant was discarded. The phage-containing

pellet was re-suspended in Tris-buffered saline and spun again for 1 min to remove residual polyethylene glycol. The cleared phage-containing supernatant was transferred to a fresh tube and stored at 4°C.

Amplified phage were titered and diluted in PBS/BSA+T to ~10^{11} PFU/mL for subsequent rounds of panning. After four rounds of panning, individual plaques from the final round were isolated and incubated overnight in LB broth containing a 1:100 dilution of an overnight culture of *E. coli* ER2738, at 37°C with gentle agitation. Phage DNA was isolated from overnight cultures using the S.N.A.P. MiniPrep kit (Thermo-Fisher Scientific) and sequenced using a -96gIII sequencing primer in order to identify the peptides displayed on the N-terminal portions of the pIII coat protein, presumably responsible for GlyR binding.

3.2.4- Two-electrode voltage-clamp electrophysiology

Oocyte isolation, injection, and electrophysiological recordings were performed as described in chapter 2 with the following modifications: All solutions were prepared in MBS or MBS + 2.5 mM tricine. Peptides were diluted in MBS or MBS + 2.5 mM tricine to final working concentrations from frozen 10 mM stocks. When maximally-effective concentrations of agonists were applied, applications lasted for 15 sec and were followed by 10-15 min washouts with MBS to allow for receptor re-sensitization. For experiments using submaximal concentrations, glycine concentrations that yielded 5-10% of the maximally-effective glycine response (EC_{5-10}) were applied for 45 sec followed by 3-5 min washouts with MBS to allow for receptor re-sensitization. Peptides were co-applied with agonist following a 45 sec pre-incubation of peptide alone.

3.2.5- Determination of Zinc Concentration in Peptide Stocks

Zinc concentrations in peptide stocks were determined using a quadrupole-based Agilent 7500ce inductively-coupled plasma mass spectrometer (ICP-MS) at the Jackson School of Geosciences Isotope Geochemistry Facility at the University of Texas at Austin. 10 mM stocks of peptide were diluted 10-fold in 2% HNO3 before analysis. Electrophysiological and ICP-MS data were collected in parallel from the same batch of each peptide.

3.3- Results

3.3.1- Selection of α1β glycine receptor-binding peptides

Phage display was conducted using the Ph.D.-7 Phage Display Library from New England Biolabs containing approximately 10^9 unique heptapeptide sequences displayed pentavalently on M13 phage. In an attempt to obtain peptides selective for the α1β GlyR subtype, we employed a modified version of our previous phage display procedure (Tipps et al., 2010) in which the phage library was first subjected to a subtractive panning step against α2β-expressing (D7.1), or α2β- and α3β-expressing (D7.2) HEK 293 cells. In total, 40 peptides were identified via phage display as capable of binding to the α1β GlyR; 15 from panning series D7.1 and 25 from D7.2 (**Table 3.1**).

Phage	Sequence
D7.1–102	SWLAMEF
D7.1–103	GGNPTIY
D7.1–104	MNPTYTM
D7.1–105	**NVAPAMS**
D7.1–106	QVRSVVH
D7.1–107	**DQTQTPR**
D7.1–109	DSKHNFS
D7.1–110	MGPPRTS
D7.1–111	TPIANPP
D7.1–112	YTSTGPA
D7.1–113	GTLWSSM
D7.1–114	**SHGRINS**
D7.1–115	HVLVPLH
D7.1–117	NMIPSHG
D7.1–118	**TDTASRS**
D7.2–011	STIHGST
D7.2–012	SIRLDSQ
D7.2–013	HAPKSDT
D7.2–014	DRMPHYF
D7.2–015	STFTKSP
D7.2–016	TGADLNT
D7.2–017	YTMPGEL
D7.2–018	VIPHVLS
D7.2–019	GVQIMGR
D7.2–110	NANAALP
D7.2–111	**SWQQGPY**
D7.2–112	GSSCCKT
D7.2–113	SILPYPY
D7.2–115	**KLPGWSG**
D7.2–116	NQLTTLN
D7.2–118	AAPTVPR
D7.2–119	QETRAPG
D7.2–120	NQLPLHA
D7.2–121	GPMLARG
D7.2–122	**TTMPIDS**
D7.2–123	**TTPTKSA**
D7.2–124	VQTYARG
D7.2–125	TSLNRYP
D7.2–126	SHTAPLR
D7.2–128	DVPVPQV

Table 3.1: List of peptide sequences identified after panning with the Ph.D-7 library.

D7.1 phage: positive selection = α1β GlyR, negative selection = α2β GlyR. D7.2 phage: positive selection = α1β GlyR, negative selection = α2β and α3β GlyR. Peptides in bold are those that enhance α1β GlyR function by ≥20% potentiation of EC5-10 glycine mediated currents.

3.3.2- Electrophysiological characterization of α1β glycine receptor-binding peptides

Of the 40 peptide sequences initially identified, 25 were synthesized and characterized for their actions on α1β GlyRs via two-electrode voltage clamp electrophysiology in *Xenopus laevis* oocytes. Peptides were tested at 30 μM, a concentration previously shown to be sufficient to affect GlyR function (Tipps et al., 2010). A glycine concentration that yielded 5 to 10 percent of the maximally-effective glycine response (EC_{5-10}) was initially identified in each oocyte and applied several times for 45 sec, followed by 3-5 min washouts to allow for receptor re-sensitization. Peptide was applied for 30 sec alone followed by a 45 sec co-application with EC_{5-10} glycine. Peptide applications resulted in a range of effects from 26.8 ± 4.9% inhibition (D7.1-115) to 93.3 ± 12.2% potentiation (D7.2-123) of the EC_{5-10} glycine response (**Fig. 3.1**). None of the peptides had effects in the absence of glycine, implying that they lack both GlyR agonist activity as well as effects at any endogenous oocyte proteins that could influence the holding current of the oocyte. During the course of conducting experiments, we sometimes noticed variations in the degrees of modulation produced by peptides synthesized in different batches (**Fig. 3.2**). This was not entirely unexpected as net-peptide content can vary between batches of synthetic peptides and peptide concentrations were calculated based on the dry weight of the lyophilized powder provided by the supplier. In order to prevent batch-to-batch variations from confounding results in characterization experiments, all data presented below were obtained from single batches of peptides.

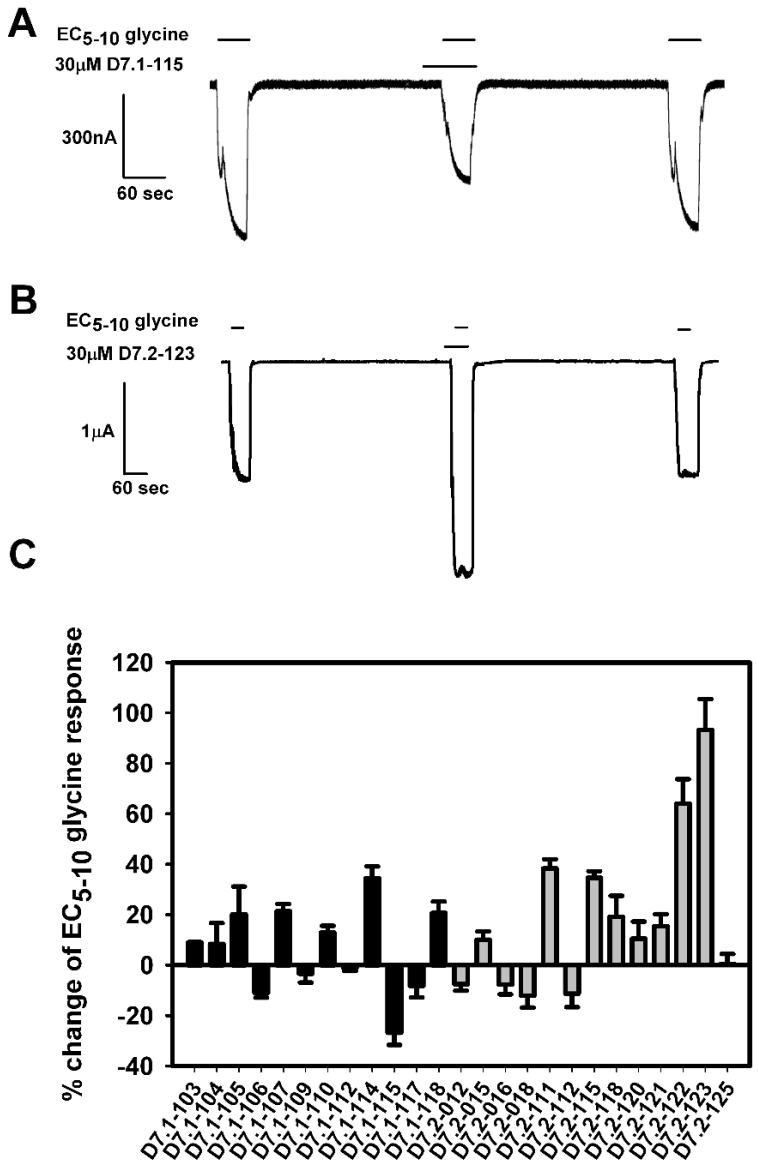

Figure 3.1: Screen for peptide activity at the α1β glycine receptor

(A) Sample two-electrode voltage-clamp tracing of the effects of 30 μM D7.1-115 on α1β GlyRs activated by EC_{5-10} glycine. (B) Sample tracing of the effects of 30 μM D7.1-122 on α1β receptors activated by EC_{5-10} glycine. (C) The percent change in EC_{5-10} glycine responses produced by 30 μM concentrations of peptides that were pre-incubated for 30 sec followed by a 45 sec co-application with EC5-10 glycine. Peptides exhibited varying degrees of activity at the α1β GlyR, but none had an effect in the absence of glycine. Data are expressed as the mean ± S.E.M. across all batches of peptides tested on 3-15 oocytes obtained from at least two frogs.

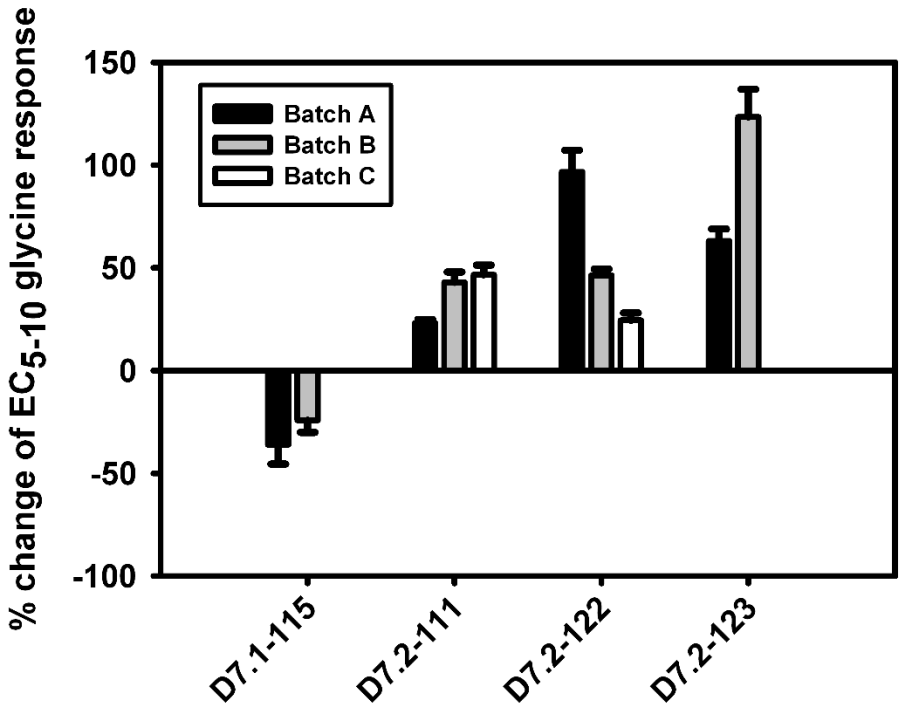

Figure 3.2: Batch-to-batch variation of peptide modulation of the α1β glycine receptor.

Four peptides were synthesized multiple times to acquire enough material for characterization experiments. The different batches displayed varying degrees of activity on α1β GlyRs when 30 μM concentrations of peptides, calculated based on dry peptide weight, were pre-incubated for 30 sec followed by a 45 sec co-applications with $EC_{5\text{-}10}$ glycine. This is likely due to varying net peptide content between batches. Data are expressed as the mean ± S.E.M. of 3-7 oocytes obtained from at least two frogs.

To determine if peptides were capable of affecting GlyR-mediated currents at lower concentrations, six different peptides of the initial screen were applied at 1, 3, 10, and 30 μM concentrations to α1β GlyRs activated by EC_{5-10} glycine. The threshold concentrations of activity for the three most efficacious of these peptides were between 3 and 10 μM (**Fig. 3.3**). Previous work in our lab illustrated the abilities of allosteric modulators to differentially affect currents produced by saturating concentrations of agonists varying in efficacy (Kirson et al., 2012, 2013). In order to test if this was also true for peptide modulation, 30 μM D7.2-122 was tested for its ability to modulate α1β GlyRs activated by maximally-effective concentrations of the partial agonist taurine and the higher efficacy agonist glycine (**Fig. 3.4**). As with other allosteric modulators, D7.2-122 had no effects on currents produced by saturating glycine concentrations but did enhance currents produced by a saturating concentration of taurine.

In order to determine whether negative selection during phage panning against GlyRs of different subunit composition allows for the identification of peptides with subunit selectivity, the most effective peptides were applied to α1β, α2β, and α3β GlyRs activated by EC_{5-10} glycine (**Fig. 3.5**). Interestingly, all peptides had higher activity on α1β and α3β receptors compared to α2β [$F(2,44) = 4.24$, $P < 0.025$; Two-way ANOVA]. However, peptides appeared to be equally or slightly more effective in enhancing α3β than α1β GlyR function, despite inclusion of the α3β receptor subunits in the D7.2 negative selection process.

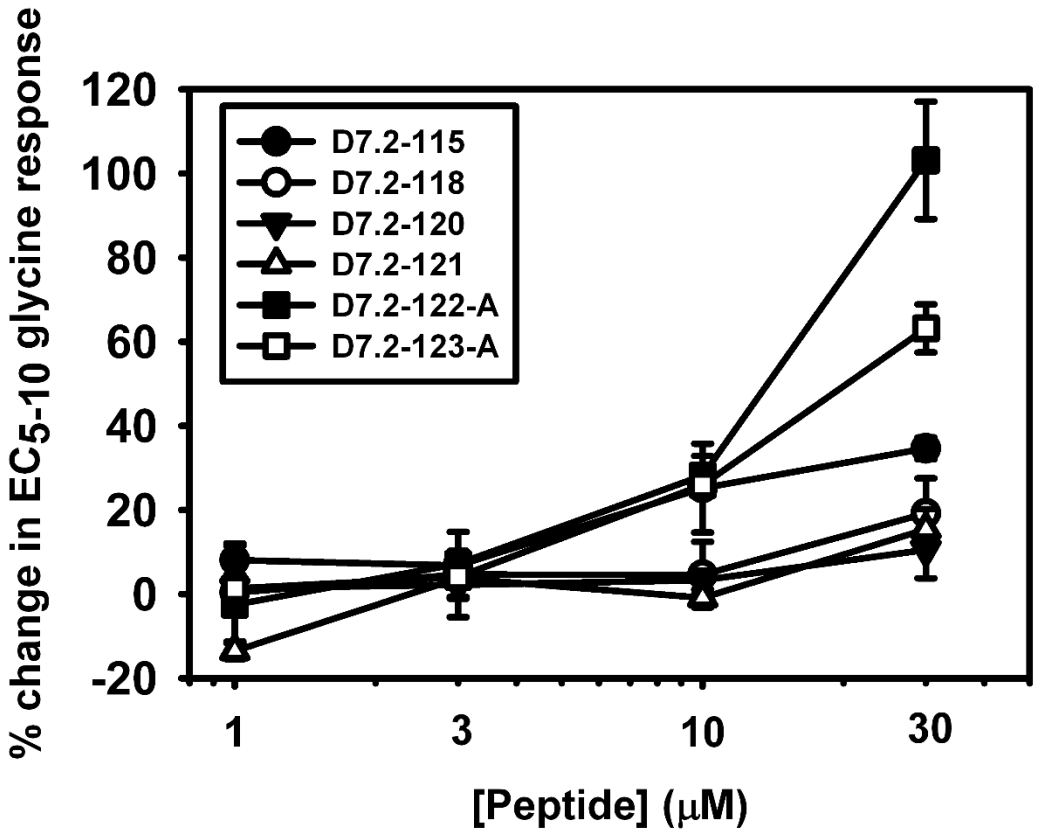

Figure 3.3: Concentration dependence of the two most effective glycine receptor enhancing peptides.

The percent change in $EC_{5\text{-}10}$ glycine currents produced by 1, 3, 10, and 30 μM concentrations of peptides pre-incubated for 30 sec followed by 45 sec co-applications with $EC_{5\text{-}10}$ glycine. Data are represented as the mean ± S.E.M. of 3-5 oocytes obtained from at least 2 frogs.

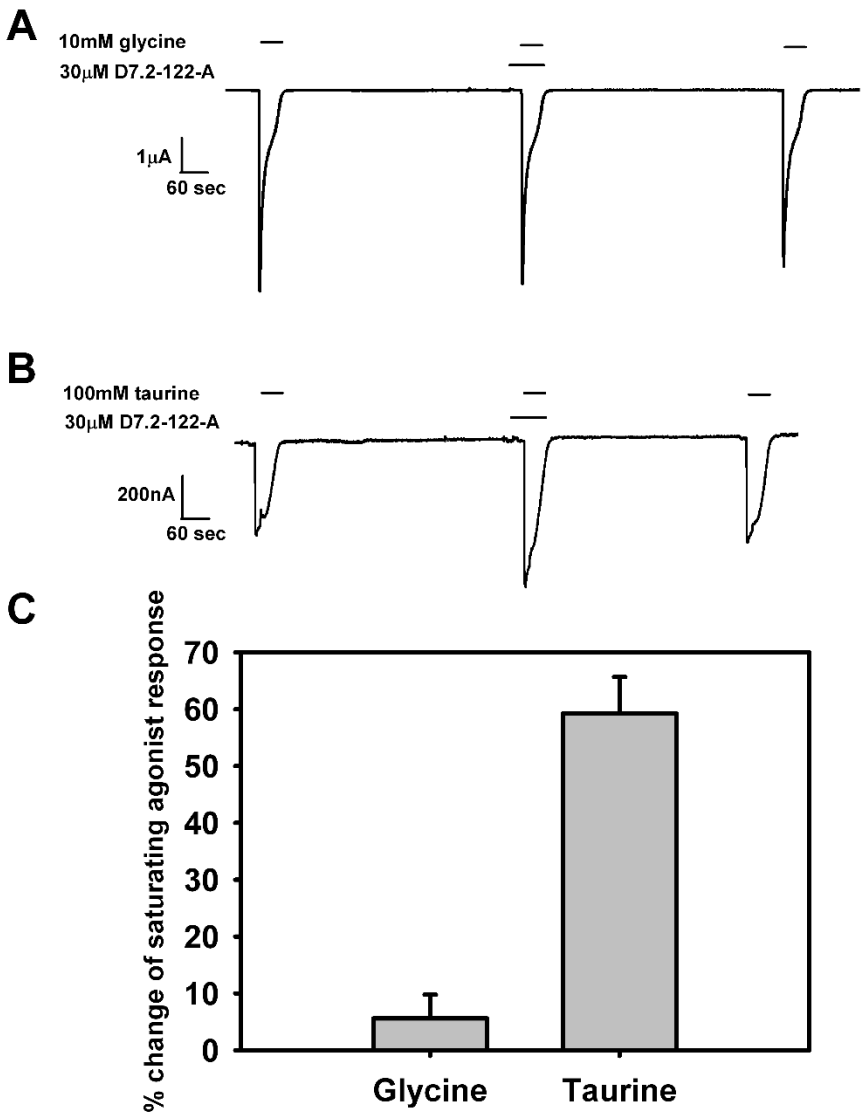

Figure 3.4: Peptide activity on α1β glycine receptors activated by maximally-effective agonist concentrations

(A) Sample tracings showing the effects of 30 μM D7.2-122-A on a saturating concentration of the high efficacy agonist glycine and (B) the partial agonist taurine. Peptide was pre-incubated 30 sec followed by a 45 sec co-application with saturating agonist concentrations. (C) Summary of the effects of 30 μM D7.2-122-A on α1β receptor currents generated by maximally effective concentrations of glycine or taurine. Peptide D7.2-122-A had no effect on currents generated by saturating glycine concentrations while enhancing currents generated by saturating taurine concentrations. Data are represented as the mean ± S.E.M. of 3 oocytes obtained from two frogs.

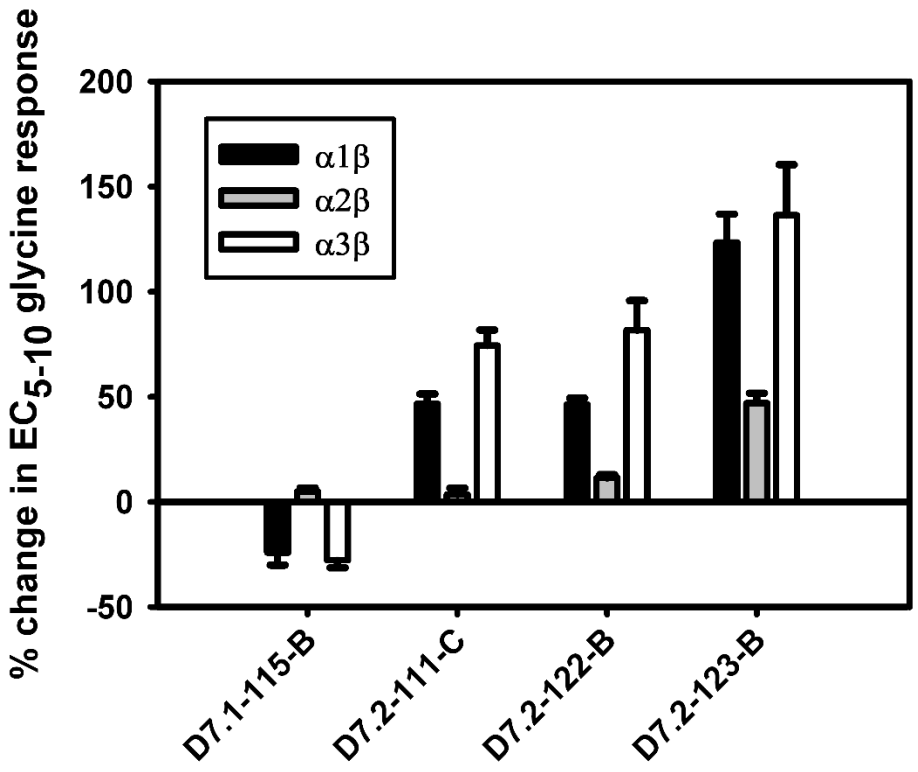

Figure 3.5: Subunit specificity of select peptides.

Percent change in $EC_{5\text{-}10}$ glycine responses on α1β, α2β, and α3β GlyRs produced by 30 sec pre-incubations of 30 μM concentrations of peptides followed by 45 sec co-applications with $EC_{5\text{-}10}$ glycine. All peptides had greater effects on α1β and α3β, compared to α2β, GlyRs. Activity at α3β receptors was the same or slightly increased for all peptides relative to α1β, despite inclusion of α3β subunits in the negative selection step of the D7.2 panning series. Data are expressed as the mean ± S.E.M. of 3-6 oocytes obtained from at least two frogs.

3.3.3- Identification of possible consensus sequences for glycine receptor-enhancing peptides

While there is no obvious amino acid consensus sequence among all of the 40 peptides identified by phage panning, analysis of their amino acid compositions revealed selection both for and against particular amino acids within the identified peptides, compared to the observed frequencies of amino acids within the library as a whole, as characterized by the manufacturer (**Fig. 3.6A,B**). **Fig. 3.6A** shows the observed frequencies of individual amino acids expressed as the percentage of all 280 amino acids present within the 40 identified heptapeptides, while **Fig. 3.6B** expresses the percent change in the observation frequency of amino acids in the 40 identified peptides compared to the observed frequency of amino acids in the whole library, as characterized by New England Biolabs. Overall, it is clear that amino acids T, P, and G were strongly selected for while K, W, F, E were selected against (≥30% increase or decrease in observed frequency, respectively). A more striking selection is seen when analyzing the amino acid compositions of only the eight GlyR-enhancing peptides that produced ≥20% potentiation of $EC_{5\text{-}10}$ glycine responses (**Table 3.1, bold; Fig. 3.6C,D**). Here T, P, S, D, Q, and W are all strongly selected for (≥45% increase in observed frequency) while L, N, V, Y, F, E, and C were strongly selected against (≥38% decrease in observed frequency).

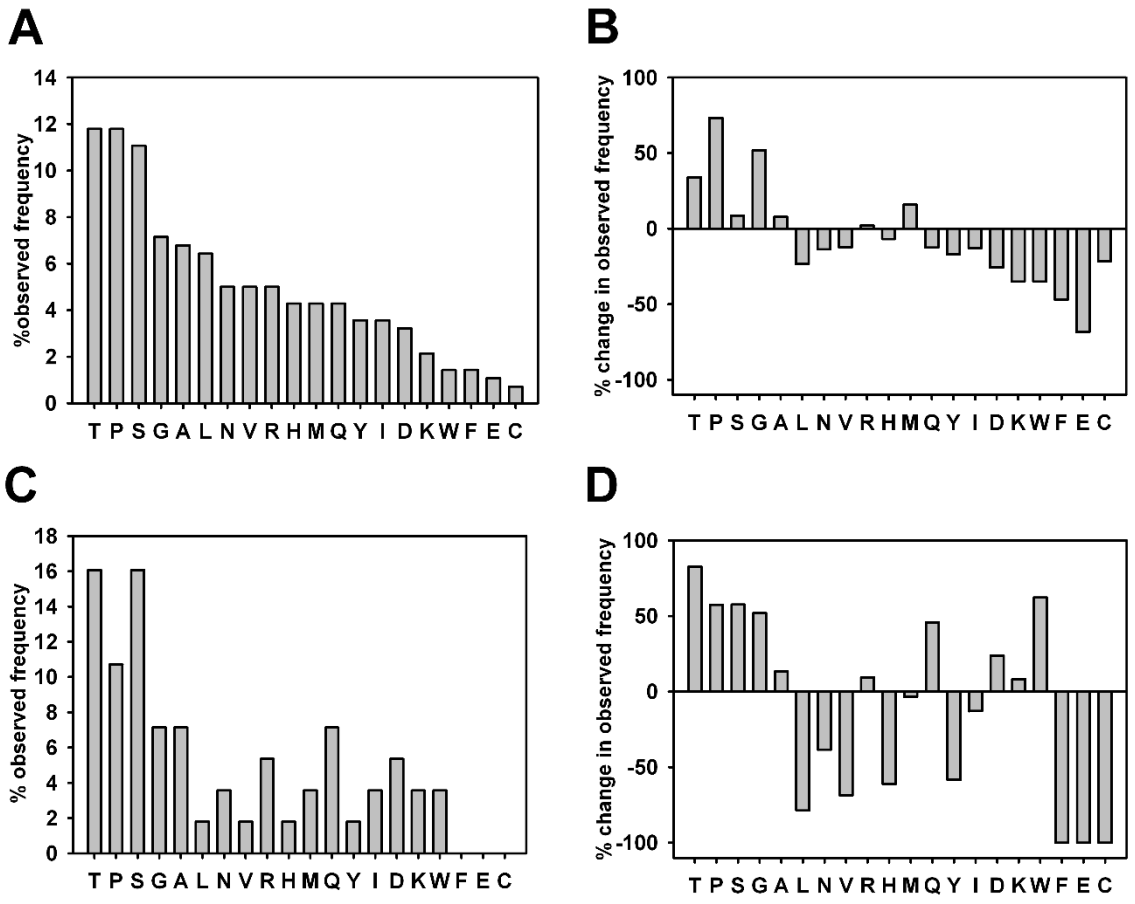

Figure 3.6: Selection for specific amino acids during phage display panning.

(A) The observed frequencies of individual amino acids expressed as the percent of all 280 amino acids within the 40 peptides identified in panning series D7.1 and D7.2. (B) The percent change in observation frequency of amino acids in the 40 identified peptides compared to the observed frequency of amino acids in the library as a whole. (C) The observed frequency of individual amino acids expressed as the percent of all 56 amino acids within the eight GlyR-enhancing peptides (those that produce ≥20% potentiation of $EC_{5\text{-}10}$ glycine mediated currents). (D) The percent change in observation frequency of amino acids in the 8 receptor enhancing peptides compared to the observed frequency of amino acids in the library as a whole. Observation frequency of amino acids in the library, as a whole, was provided by NEB.

Peptide	Sequence
Motif	**P**xx**S**
D7.1-105	NVA**P**AM**S**
D7.2-115	KL**P**GW**S**G
D7.2-122	TTM**P**ID**S**
D7.2-123	TT**P**TK**S**A
Motif	**T**x**P**xx**S**
D7.2-122	T**T**M**P**ID**S**
D7.2-123	**T**T**P**TK**S**A
Motif	xxx**Q**x**P**x
D7.1-107	DQT**Q**T**P**R
D7.2-111	SWQ**Q**G**P**Y
Motif	**T**x**T**
D7.1-107	DQ**T**Q**T**PR
D7.1-118	**T**D**T**ASRS
D7.2-123	T**T**P**T**KSA
Motif	**TT**xxxxx
D7.2-122	**TT**MPIDS
D7.2-123	**TT**PTKSA

Table 3.2: Possible consensus motifs for α1β GlyR enhancing peptides (those that produce ≥20% potentiation of EC5-10 glycine mediated currents).

Further analysis of the positions of the amino acids that were strongly selected for in the eight GlyR-enhancing peptides revealed several possible consensus sequences that may contribute to their enhancing abilities (**Table 3.2**). First, serines are present towards the C-terminal regions of peptides, at positions 6 or 7 in six of eight enhancers. Further, four out of eight peptides contain the sequence PxxS and in the two most effective peptides, D7.2-122 and D7.2-123, this sequence is expanded to TxPxxS, where 'x' could be any amino acid. The two peptides that do not contain serines at the C-terminal region (D7.1-107 and D7.2-111) contain the consensus xxxQxPx. In addition, D7.1-107, D7.1-118, and D7.2-123 all contain TxT while D7.2-122 and D7.2-123 both contain TTxxxxx as well as sharing the sequence TxPxxS. In order to determine if consensus data could be used in the design of custom GlyR-enhancing peptides, we generated a synthetic peptide combining the TTxxxxx and TxPxxS motifs of D7.2-122 and D7.2-123. This custom peptide, denoted Con-1, had the sequence TTAPAAS and enhanced $\alpha 1\beta$ GlyR function in a concentration-dependent manner (**Fig. 3.9**)

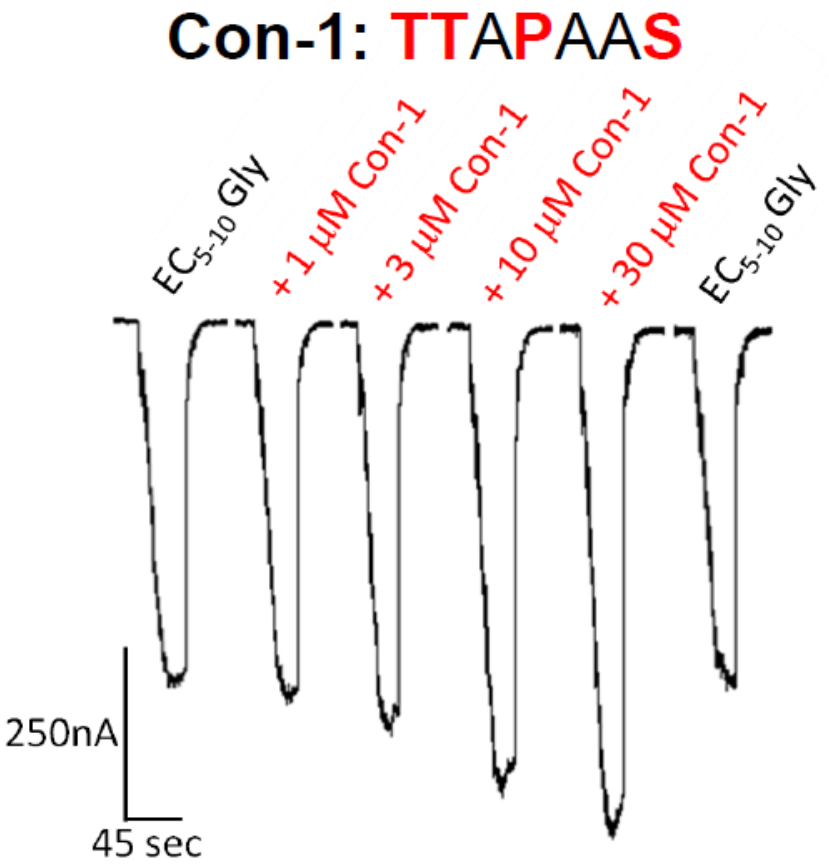

Figure 3.7: Concentration-dependent enhancement of α1β GlyR function by custom designed peptide.

Peptide Con-1 (TTAPAAS) was designed through the combination of the consensus sequences found in the two most effective GlyR-enhancing peptides. Con-1 enhanced EC_{5-10} glycine currents in a concentration dependent manner, validating the use of the consensus sequence data in the development of custom GlyR-enhancing peptides. Tracing representative of two independent experiments.

3.3.4- Characterizing the role of zinc in peptide action

Zinc affects modulation of the GlyR by ethanol, with zinc chelation markedly decreasing alcohol enhancement of receptor function when tested using submaximal glycine concentrations (McCracken et al., 2010). In order to determine what role, if any, zinc plays in the activities of these peptides at the GlyR, potentiating concentrations of select peptides were co-applied with the zinc-chelating agent tricine. As seen in **Fig. 3.8**, removal of zinc by tricine almost completely abolishes modulation by all peptides except D7.2-123. Interestingly, the inhibitory action of D7.1-115 was also blocked in the presence of tricine, implying a possible zinc-dependence of peptide action. Moreover, zinc is a known contaminant in many biological buffers and labware at concentrations capable of affecting GlyR function (Cornelison and Mihic, 2014; Kay, 2004). To ensure that the variable effects of the different peptides we tested were not due to varying levels of zinc contamination within the peptides themselves, samples of a variety of peptides were submitted for ICP-MS analysis to quantify total zinc content. **Fig. 3.9** compares peptide effects on $EC_{5\text{-}10}$ glycine-mediated currents relative to zinc concentrations measured from those specific peptide stocks. No correlation between the two measures was seen.

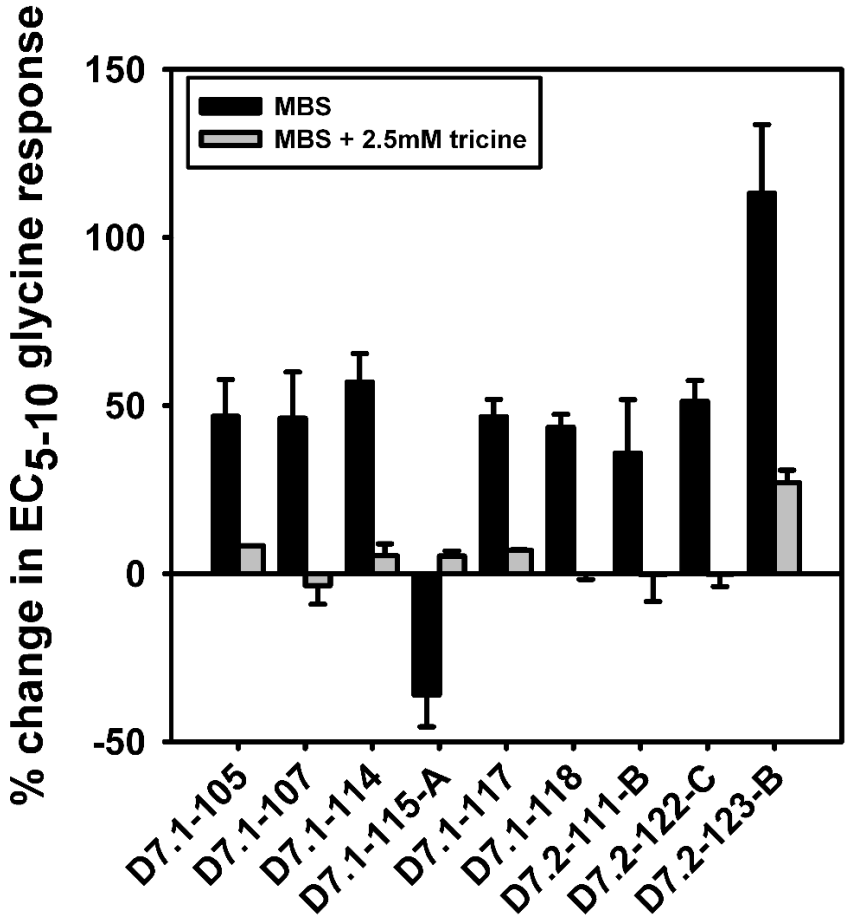

Figure 3.8: Zinc chelation by tricine inhibits the effects of peptides at α1β glycine receptors.

The percent change in $EC_{5\text{-}10}$ glycine responses produced by peptides that were pre-incubated for 30 sec followed by a 45 sec co-application with $EC_{5\text{-}10}$ glycine in MBS or MBS + 2.5 mM tricine. All peptides were applied at a concentration of 100 μM except D7.2-111-A and D7.2-123-B which were applied at 30 μM. Zinc chelation by tricine blocked or strongly inhibited the effects of all peptides tested. Data are expressed as the mean ± S.E.M. of 3-4 oocytes obtained from at least two frogs.

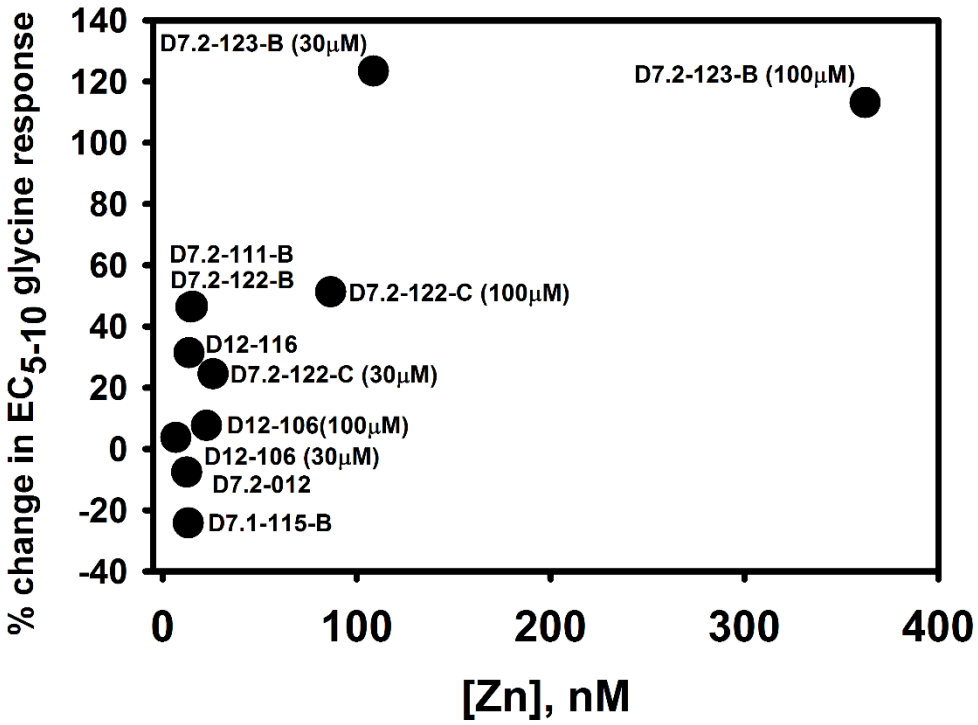

Figure 3.9: Correlation between peptide activity on α1β receptors and zinc contamination as determined by ICP-MS of peptide solutions.

Aliquots of the same batch of peptide were used for the determination of GlyR modulation, via two-electrode voltage clamp, and quantitation of total zinc content in peptide stock by ICP-MS. Electrophysiological and ICP-MS data represent the values for 30 μM peptide except where noted, when multiple concentrations were tested. The symbols for peptides D7.2-111-B and D7.2-122-B overlap.

3.4- Discussion

The GlyR is a putative target for the treatment of inflammatory pain and alcoholism (Blednov et al., 2015; Molander et al., 2007, 2005; Molander and Söderpalm, 2005a; Tipps et al., 2010; Xiong et al., 2012) and novel specific modulators of GlyR function could thus have potential therapeutic applications. Of particular interest would be modulators capable of enhancing receptor activity, as several studies have shown that GlyR activation in the nucleus accumbens and ventral tegmental area decreases ethanol drinking in rodents (Molander and Soderpalm, 2005a; Molander et al., 2005; Li et al. 2012). Tipps et al. (2010) demonstrated the feasibility of utilizing phage display to identify novel peptide modulators of the GlyR. I expanded on these studies, identifying heptapeptides acting on the GlyR with subunit selectivity (**Figs. 3.1 & 3.5**), and characterizing peptide actions on receptor function. To achieve this, I incorporated a negative selection step into the phage selection process, in which the phage library was first washed over HEK 293 cells expressing GlyR subunits we wished to select against before panning against the GlyR target of interest. Peptides that bound non-specifically to the endogenous membrane components of HEK 293 cells, or to our expressed negative selection receptor subunits would, in principle, be eliminated from the population of the library upon transfer of the supernatant to the positive selection plates containing HEK 293 cells expressing the $\alpha1\beta$ GlyR. Through this negative selection step peptides capable of acting selectively on $\alpha1\beta$ compared to $\alpha2\beta$ GlyRs were identified. However, we did not identify peptides that differentiated between $\alpha1\beta$ and $\alpha3\beta$ GlyRs (**Fig. 3.5**) despite the inclusion of the latter in the negative selection procedure of panning series D7.2. This may be due to a higher level of similarity between $\alpha1$- and $\alpha3$-containing GlyRs at the peptide binding areas. Why then did any $\alpha3\beta$-binding peptides survive the

panning procedure if they were negatively selected against? One reason could be that the panning is somewhat 'leaky'. If some phage capable of binding in the negative selection do not bind, but do bind in the subsequent positive selection, they would be amplified and re-introduced into the next round of panning.

For molecules with potential for therapeutic use, high potency is desired, preferably in the nM or lower concentration range. However, the GlyR-specific peptides identified using the New England Biolabs Ph.D. libraries, both in this study (**Fig. 3.3**) and by Tipps et al. (2010), acted at low μM concentrations. This could be due to the pentavalent display of these libraries. Multivalent display can lead to an avidity effect where phage displaying multiple copies of a particular peptide, each binding to the target, increase the apparent affinities of the multivalent phage-peptide fusions, compared to when peptides are tested alone. This phenomenon has been well documented in the phage display literature (Lowman, 1997). In order to overcome this, one can utilize a monovalent display system, such a phagemid display system, when performing panning procedures (Lowman, 1997; Qi et al., 2012). Alternatively, peptide potency could be increased through affinity maturation by using the sequence of the most promising peptides from initial screens, such as D7.2-122 or D7.2-123 in this case, as a scaffold for the creation of a new phage library in which all displayed peptides would be some variation of the originally-identified sequences. We are investigating both possible methods to identify peptides that act with greater potency at the GlyR.

The variety of amino acid sequences seen in the 40 identified peptides (Table 3.1) reflects the large and diverse nature of the pentameric GlyR, as it is likely that such a large molecular structure presents many potential binding sites for the various peptides contained within the library. Further, it should be noted that phage display can only identify peptides capable of interacting with a particular target, and does not provide

information about any possible functional effect of the peptides at that target. Therefore, we narrowed our consensus search to only the eight peptides that enhanced $EC_{5\text{-}10}$ receptor currents ≥20% and revealed several possible amino acid consensus sequences that may contribute to their abilities to positively modulate GlyR function (Table 3.2). The most prevalent consensus was the sequence PxxS, found in four of eight peptides, where the proline residue is located at either positions 3 or 4 and the serine is located at the C-terminal positions 6 or 7. Further, this consensus is expanded within the top two enhancing peptides, D7.2-122 and D7.2-123, to TxPxxS. These two peptides also both contained TTxxxxx, which may also be responsible for their robust abilities to enhance GlyR function. In addition to these sequences, we found that three of eight allosteric enhancers contained the sequence TxT, and two contained xxxQxPx. Of the 17 of 25 characterized peptides that did not enhance receptor function ≥20%, TxT was found in three (D7.1-104, D7.1-112, and D7.2-015), QxP was found in one (D7.2-120) and PxxS was found in one (D7.1-110). It may be that these three consensus sequences allow for interaction with the GlyR, and enhancing activity relies on the presence of other specific sequences within the peptide; alternatively, other amino acids within these peptides may interfere with the GlyR-enhancing activity that these consensus sequences contribute. Further, it is possible that these peptides could enhance receptor activity at higher concentrations, but are less potent than the eight that did.

We next asked whether consensus data could be used in the design of custom GlyR-enhancing peptides. In order to maximize the probability of success, we combined the consensus sequences that were shared by the two most effective GlyR-enhancing peptides, D7.2-122 and D7.2-123. The resultant peptide, denoted Con-1, had the sequence TTAPAAS and was capable of enhancing α1β GlyR function in a concentration dependent manner with approximately 40% enhancement by 30 μM

peptide (**Fig. 3.7**). This demonstrates the utility of using the consensus sequences reported here as a starting point in the future design of higher potency peptides. One potential method for such studies could be the creation of a novel phage display library in which each clone displays a variant of the Con-1 sequence, replacing the alanine residues with a variety of different amino acids. This type of phage display based affinity maturation could allow for the identification of complementary residues that increase the ability of the core consensus sequence to enhance GlyR function.

Zinc is a biphasic modulator of the GlyR, capable of enhancing receptor function at concentrations below approximately 10 μM, and inhibiting it at higher concentrations (Bloomenthal et al., 1994; Harvey et al., 1999; Laube et al., 1995). It is clear that some peptides, such as D7.2-123-B, contain sufficient contaminating zinc to affect GlyR function. However, ICP-MS measures total zinc, and there is no way to tell how much of this value reflects free zinc concentrations acting at the GlyR. Further, the relationship between the degree of receptor modulation observed and zinc concentration of the various peptides indicates that peptide effects cannot solely be due to contaminating zinc (**Fig. 3.9**). For example, peptides that range in activity between -24.19% to 46.60% change in $EC_{5\text{-}10}$ glycine responses contain between 12.38 - 15.36 nM zinc, a very small range that could not account for the degree of variance observed in the peptide effects. Further, D7.2-123-B had very similar effects at 30 and 100 μM concentrations, despite there being over a 3-fold difference in zinc concentration (108.68 vs. 362.38 nM zinc) in those two peptide preparations. GlyR potentiation by zinc typically peaks around 1 μM zinc (Miller et al., 2005), with significantly different effects between 100 and 300 nM zinc. Therefore, the similarity in effects between the different concentrations of D7.2-123-B must be due to some interaction between both zinc and peptide effects at the GlyR.

Previous studies have shown that zinc interacts with other allosteric modulators at the GlyR (Kirson et al., 2013; McCracken et al., 2010, 2013a,b). Of particular note, is that the chelation of zinc via tricine decreases the effects of alcohol at α1-, α2-, and α3-containing GlyRs, essentially blocking the actions of ethanol at concentrations producing under 100% potentiation of EC_{5-10} glycine currents (≤100 mM ethanol), while significantly inhibiting alcohol effects at higher concentrations (200 mM ethanol) (McCracken et al., 2010, 2013a,b). It is possible that zinc acts as a co-factor necessary to observe appreciable modulation of receptor function by allosteric modulators at concentrations that produce little effect on their own. Nanomolar concentrations of zinc sufficient to enhance GlyR function are ubiquitous *in vivo* and *in vitro*, and the receptor may have evolved to respond optimally to glycine when zinc is bound. Thus it is probable that peptide selection occurs to zinc-bound GlyRs during the panning procedure. Regardless, free or rapidly-exchangeable forms of zinc are maintained in the brain at levels capable of potentiating receptor currents, indicating that zinc dependence would not necessarily hinder the potential use of these peptides *in vivo* (Frederickson et al., 2006b; Frederickson and Bush, 2001).

In summary, we have extended our initial observations demonstrating the utility of using a modified phage display procedure in the discovery of novel allosteric modulators of the GlyR. Expression of target channels in HEK 293 cells ensured the proper conformation of multimeric channels for phage display libraries to be panned against, yielding numerous heptapeptide sequences. The addition of a negative selection step in which libraries are first washed over cells expressing receptors similar to the target can help in obtaining peptides with subunit selectivity, but may require a more stringent selection if the targets are molecularly similar. Additionally, the zinc-dependence of peptide activity seen here (**Fig. 3.8**), taken together with previous

observations of the zinc-dependence of ethanol actions on the GlyR, indicates a possible function of zinc as an endogenous co-modulator necessary for efficient allosteric modulation of this receptor by some modulators.

Chapter 4: Validating the use of mutant glycine receptors for studying interactions between allosteric modulators and zinc

4.1- Introduction

A variety of agents act as positive allosteric modulators of GlyR function, including ethanol, volatile anesthetics, and inhaled drugs of abuse (Mihic et al., 1997; Beckstead et al., 2000; Welsh et al., 2010). In addition to these exogenous compounds, GlyRs are also modulated by endogenous agents such as the divalent metal cation zinc (Bloomenthal et al., 1994; Laube et al., 1995). Rapidly-exchangeable zinc (referred to as "free" zinc) is ubiquitous in the central nervous system, found both tonically at nanomolar concentrations in cerebrospinal fluid (CSF) (Frederickson et al., 2006b) and released phasically from zinc-containing synapses, resulting in higher local concentrations (Frederickson et al., 2006a; Qian and Noebels, 2005; Vogt et al., 2000). Zinc biphasically modulates GlyR function, enhancing currents at concentrations below ~10μM while inhibiting GlyR function at higher concentrations (Bloomenthal et al., 1994; Laube et al., 1995). This is thought to be the result of at least two distinct zinc-binding sites on the receptor, a high affinity GlyR-enhancing site, and a low affinity inhibitory site (Harvey et al., 1999; Millet et al., 2005; Nevin et al., 2003). While tonic free zinc is found at concentrations on the lower end of the GlyR-enhancing range (Frederickson et al., 2006b), the concentrations of zinc released synaptically are still somewhat controversial. Many studies report that local zinc concentrations remain within the GlyR-enhancing range (<10μM) after synaptic release, while other studies have measured concentrations of up to 100 μM zinc (Frederickson et al., 2006a; Qian and Noebels, 2005; Vogt et al., 2000).

In addition to being present *in vivo*, zinc is a contaminant commonly found in many types of labware and reagents at nanomolar concentrations capable of affecting GlyR function (Cornelison and Mihic, 2014; Kay, 2004). Additionally, chelation of contaminating zinc significantly decreases GlyR enhancement by a variety of modulators, including ethanol (McCracken et al., 2010, 2013a,b) and recently-discovered synthetic peptides (See chapter 3; Cornelison et al., 2016). However, the mechanism by which zinc interacts with these other allosteric modulators at the GlyR is currently unknown. Further, these studies have relied on the use of relatively high (2.5 - 10 mM) concentrations of the zinc-chelator tricine that could conceivably affect channel or modulator function in ways apart from its ability to chelate zinc. For example, it is possible that tricine could act itself as a negative allosteric modulator of GlyR function and that the reduction in GlyR currents seen in the presence of tricine could be due to this effect in addition to chelation of zinc. Additionally, the strong hydrogen bonding potential of tricine could effectively "chelate" hydrophilic allosteric modulators of the GlyR, such as ethanol, thereby seemingly decreasing their effects. Further, tricine's high ionic strength could conceivable alter peptide conformation, thereby accounting for the tricine-mediated inhibition of peptide modulation seen in chapter 3.

Several single point mutations of the α1 GlyR have been reported to confer insensitivity to enhancement by zinc, including substitutions of the aspartic acid residue at position 80 to alanine (D80A) (Hirzel et al., 2006; Lynch et al., 1998; McCracken et al., 2013a) or glycine (D80G) (Laube et al., 2000), as well as the mutation of tryptophan at position 170 to serine (W170S) (Zhou et al., 2013). In this chapter we investigate the utility of these "zinc-insensitive" GlyR mutants as an alternative method to zinc chelation for the study of GlyR modulation in the absence of co-modulation by zinc.

4.2- Materials and Methods

4.2.1- Generation of point mutations

The α1 D80A GlyR mutant cDNA was a gift from the lab of Dr. R. Adron Harris (McCracken et al., 2013a). The α1 D80G and W170S point mutations were generated via site-directed mutagenesis with the QuickChange II mutagenesis kit (Agilent Technologies, Santa Clara, CA) and commercially engineered mutagenesis primers (Integrated DNA Technology, San Diego, CA) using wildtype α1 GlyR cDNA in a modified pBK-cytomegalovirus vector (Mihic et al., 1997) as a template. Successful mutagenesis was verified via Sanger sequencing using AB 3730 and AB 3730XL DNA analyzers (Thermo-Fisher Scientific, Waltham, MA) at the University of Texas at Austin DNA Sequencing Facility. The GlyR α1 cDNAs were completely sequenced to ensure the absence of any unwanted mutations.

4.2.2- Two-electrode voltage-clamp electrophysiology

Oocyte isolation, injection, and electrophysiological recordings were performed as described in chapter 2. Glycine and modulators were diluted in, and all electrophysiological recordings were performed in, modified Barth's saline (MBS) buffer. For all experiments, modulators were pre-incubated for 30 sec prior to co-application with glycine. When maximally-effective concentrations of glycine were used, applications lasted for 10 sec and were followed by a 15 min (wildtype and W170s GlyRs) or 20 min (D80A and D80G GlyRs) washout to allow for full receptor re-sensitization. When concentrations of glycine giving 5-10% of a maximally effective concentration of glycine (EC_{5-10}) were used, applications lasted 45 sec followed by a 5 minute washout. For all experiments using EC_{5-10} glycine, maximally effective glycine responses were determined and used to find the EC_{5-10} glycine. This process was

repeated for each condition tested (i.e. MBS, MBS + 2.5 mM tricine). Every EC_{5-10} glycine + modulator co-application was flanked by an EC_{5-10} glycine control for comparison. Maximally effective glycine responses were checked again at the end of every experiment to account for drift in glycine responses over time. Applications in which control EC_{5-10} glycine responses drifted above EC_{10} or below EC_5 were not used. For zinc concentration-response curves, the effects of 0.03-10 μM $ZnCl_2$ on EC_{5-10} glycine currents were determined. Washout durations after zinc co-application had to be increased from 5 min to 10-15 min when concentrations > 300 nM $ZnCl_2$ were used in order to allow for complete zinc washout to occur and for return to baseline EC_{5-10} currents. For glycine concentration-response curves, 0.03-100 mM glycine (in MBS + 2.5 mM tricine, with or without 2.5 μM $ZnCl_2$), were applied for 10-45 sec as required to reach stable peak currents.

4.2.2 Data analysis

Peak currents were measured and used in data analysis. For each oocyte, currents observed in the presence of glycine plus modulators were compared with currents generated by glycine alone and expressed as the mean ± S.E.M of the percent change in glycine-mediated current. Significant differences were determined using the Students t-test, analysis of variance (ANOVA), two-way ANOVA, and Tukey post-hoc tests, as indicated. All statistical testing was performed using SigmaPlot 11.0 (Systat Software, San Jose, CA).

4.3- Results

4.3.1- Characterization of α1 wildtype, D80A, D80G, and W170S GlyR sensitivity to enhancing concentrations of zinc.

In order to verify previously-published data reporting that D80A, D80G, or W170S mutations of the α1 GlyR confer insensitivity to enhancing concentrations of zinc, mutant and wildtype homomeric receptors were expressed in *Xenopus laevis* oocytes. The effects of 100 nM $ZnCl_2$ and 2.5 mM of tricine were determined on currents generated by a concentration of an EC_{5-10} concentration of glycine. Surprisingly, in addition to wildtype receptors, D80A and D80G GlyRs showed robust sensitivity to both contaminating and exogenously-added zinc (**Fig. 4.1**). **Figs. 4.1A** and **4.1B** show that tricine chelation of contaminating zinc, previously reported to measure around 45 nM (Cornelison and Mihic, 2014), resulted in a similar decrease in EC_{5-10} glycine currents in wildtype, D80A, and D80G receptors while minimally affecting W170S GlyRs. **Figs. 4.1A** and **4.1C** show that the co-application of 100 nM $ZnCl_2$ caused a similar enhancement of EC_{5-10} glycine currents in wildtype and D80A GlyRs that was significantly reduced in D80G and abolished in W170S GlyRs.

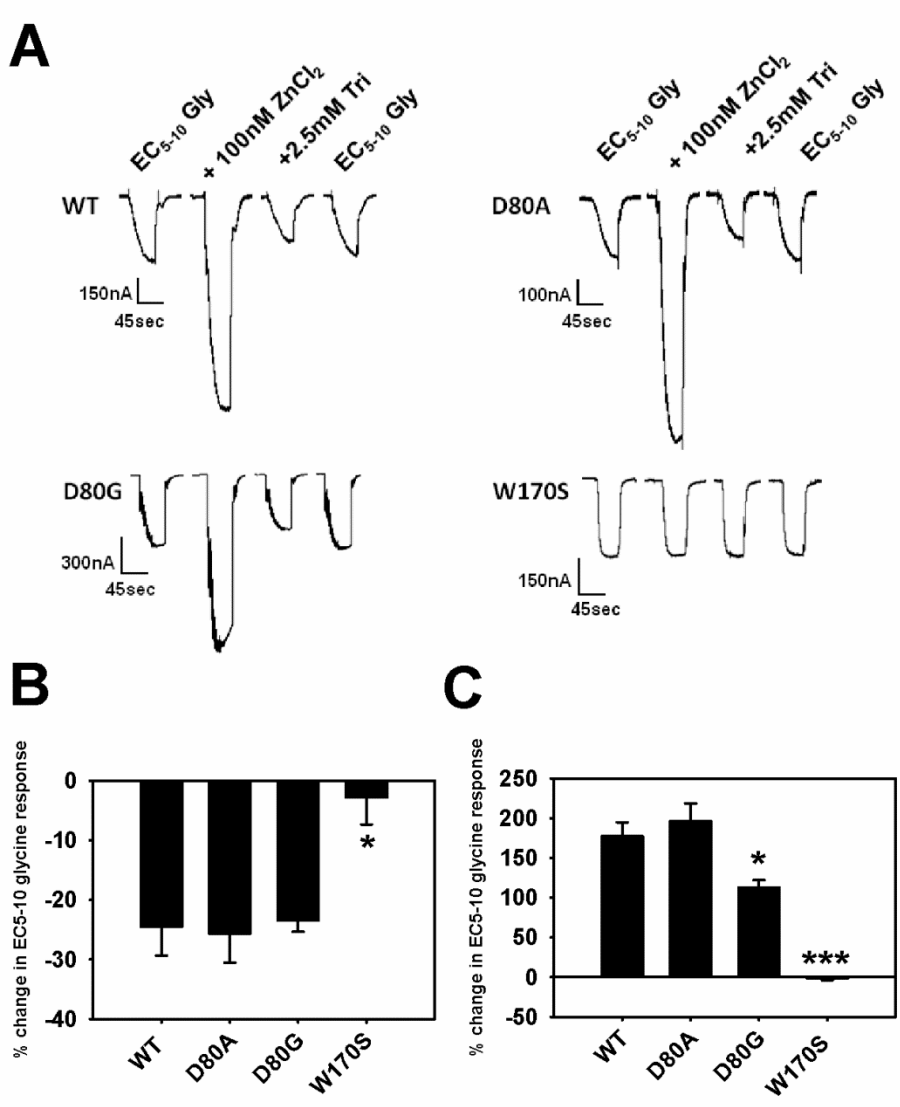

Figure 4.1: The effects of low nanomolar concentrations of zinc on α1 homomeric wildtype (WT), D80A, D80G, and W170S glycine receptors.

(A) Sample tracings showing the effects of 100 nM $ZnCl_2$ or 2.5 mM of the zinc-chelator tricine on EC_{5-10} glycine responses in WT or mutant α1 glycine receptors. (B) Summary graph of the effects of 2.5 mM tricine on EC_{5-10} glycine currents in WT and mutant glycine receptors. Tricine had a significantly decreased effect on W170S compared to WT GlyRs. Data are shown as the mean ± S.E.M. of 3-4 oocytes. One-way ANOVA followed by Tukey's post-hoc: *, $P < 0.05$. (C) Summary graph of the effects of 100 nM $ZnCl_2$ on EC_{5-10} glycine currents in WT and mutant GlyRs. 100 nM $ZnCl_2$ had a significantly decreased effect on D80G and W170S compared to WT GlyRs. Data are shown as the mean ± S.E.M of 9-16 oocytes obtained from at least two frogs. One-way ANOVA followed by Tukey's post-hoc tests: *, $P < 0.05$. ***, $P < 0.001$.

Due to the unexpected findings of robust enhancement by low nanomolar concentrations of zinc in α1 GlyR D80 mutants that were previously reported as being zinc-insensitive, we performed a more complete characterization of zinc enhancement in both wildtype and mutant receptors. We first examined zinc-sensitivity of EC_{5-10} glycine currents across a wide range of GlyR-enhancing concentrations. **Fig. 4.2** illustrates the difference in GlyR enhancement of wildtype, D80A, D80G, and W170S GlyR by co-application of EC_{5-10} glycine with 30 nM to 10 μM $ZnCl_2$. A two-way ANOVA revealed a significant effect of genotype [$F(3,119) = 76.68$, $p < 0.001$], a significant effect of zinc concentration [$F(5,119) = 14.367$, $p < 0.001$], and a significant interaction of genotype x zinc concentration [$F(15,119) = 2.40$, $p < 0.01$]. Wildtype, D80A, and D80G receptors displayed bimodal zinc-response curves where zinc-enhancement increased in a concentration-dependent manner until peak potentiation was reached at 1 μM. This was followed by a concentration-dependent decrease in zinc enhancement that reflects zinc binding to the low-affinity inhibitory sites, resulting in competition between zinc-enhancement and inhibition. Conversely, W170S GlyRs showed no zinc-enhancement from 30 nM to 1 μM $ZnCl_2$, with inhibition at higher concentrations, consistent with previous results (Zhou et al., 2013). When comparing wildtype to D80 mutant GlyRs, all three receptors had similar levels of enhancement at the low and high ends of the curve. However, D80G GlyRs exhibited a shallower zinc concentration-response curve than wildtype receptors, with significantly decreased peak zinc enhancement ($p < 0.05$). D80A GlyRs appeared to have a steeper curve with higher peak zinc-enhancement, but this was not statistically significant ($p = .120$).

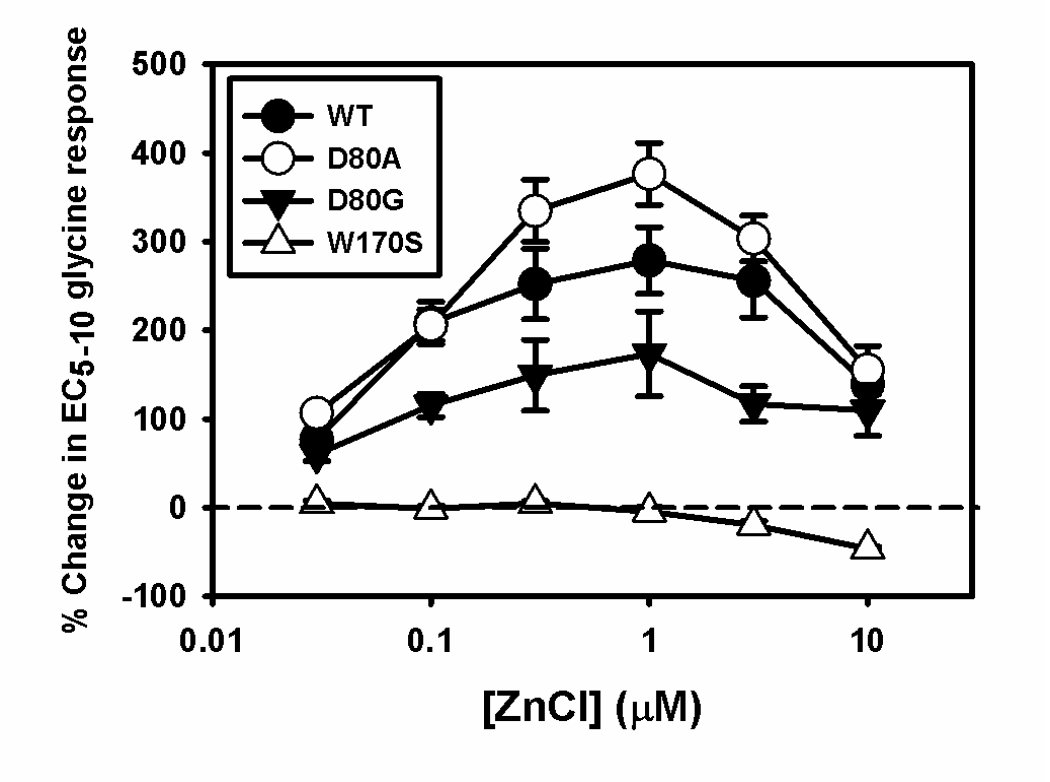

Figure 4.2.: The effects of enhancing concentrations of $ZnCl_2$ on α1 homomeric WT, D80A, D80G, and W170S glycine receptors activated by EC5-10 glycine.

Data are shown as the mean ± S.E.M. of 4-8 oocytes obtained from at least two frogs. A two-way ANOVA showed a significant effect of genotype [$F(3,119) = 76.68$, $p < 0.001$], a significant effect of zinc concentration [$F(5,119) = 14.367$, $P < 0.001$], and a significant interaction between genotype and zinc concentration [$F(15,119) = 2.402$, $p < 0.005$]. WT, D80A, and D80G GlyR show bimodal concentration response curves with peak enhancement at 1 μM $ZnCl_2$. Tukey's post-hoc tests revealed peak zinc enhancement was significantly decreased in D80G compared to WT GlyR ($p < 0.05$) and while peak enhancement appeared to be increased in D80A compared to WT GlyR, this was not significant ($p = 0.12$). W170S GlyR currents were unaffected by $ZnCl_2$ up to 1 μM and inhibited by higher concentrations.

We next investigated how zinc enhancement of receptor function changes with increasing agonist concentration. We first generated glycine concentration-response curves in the presence of 2.5 mM tricine to account for the effects of contaminating zinc on agonist EC. We then plotted glycine EC values (triangles, right ordinate) and potentiation of corresponding currents by application of 2.5 μM $ZnCl_2$ in the presence of 2.5 mM tricine (circles, left ordinate) against glycine concentration (**Fig. 4.3**). Zinc potentiation significantly decreased with increasing glycine concentration for wildtype, D80A, and D80G receptors while W170S GlyRs showed minimal zinc potentiation that did not vary with agonist concentration. When zinc potentiation of wildtype and D80 mutant GlyRs was plotted against glycine EC (**Fig. 4.4**), there appeared to be differences between wildtype and mutant GlyRs in the magnitude of zinc enhancement at lower glycine ECs (< EC_{40}). In an attempt to quantify this, we partitioned the data into zinc effects at low (EC_{0-20}) and intermediate-low (EC_{20-40}) glycine. One-way ANOVAs revealed significant differences between genotype at both low [$F(2,26) = 5.812$, $p < 0.01$] and intermediate-low EC [$F(2,9) = 5.886$, $p < 0.05$]. Tukey multiple comparisons post-hoc tests showed that compared to wildtype receptors, zinc-enhancement of D80G receptors was significantly decreased at intermediate-low (EC_{20-40}) glycine (+38.70 ± 11.64% change for D80G compared to +92.07 ± 8.91% change for WT, $p < 0.01$).

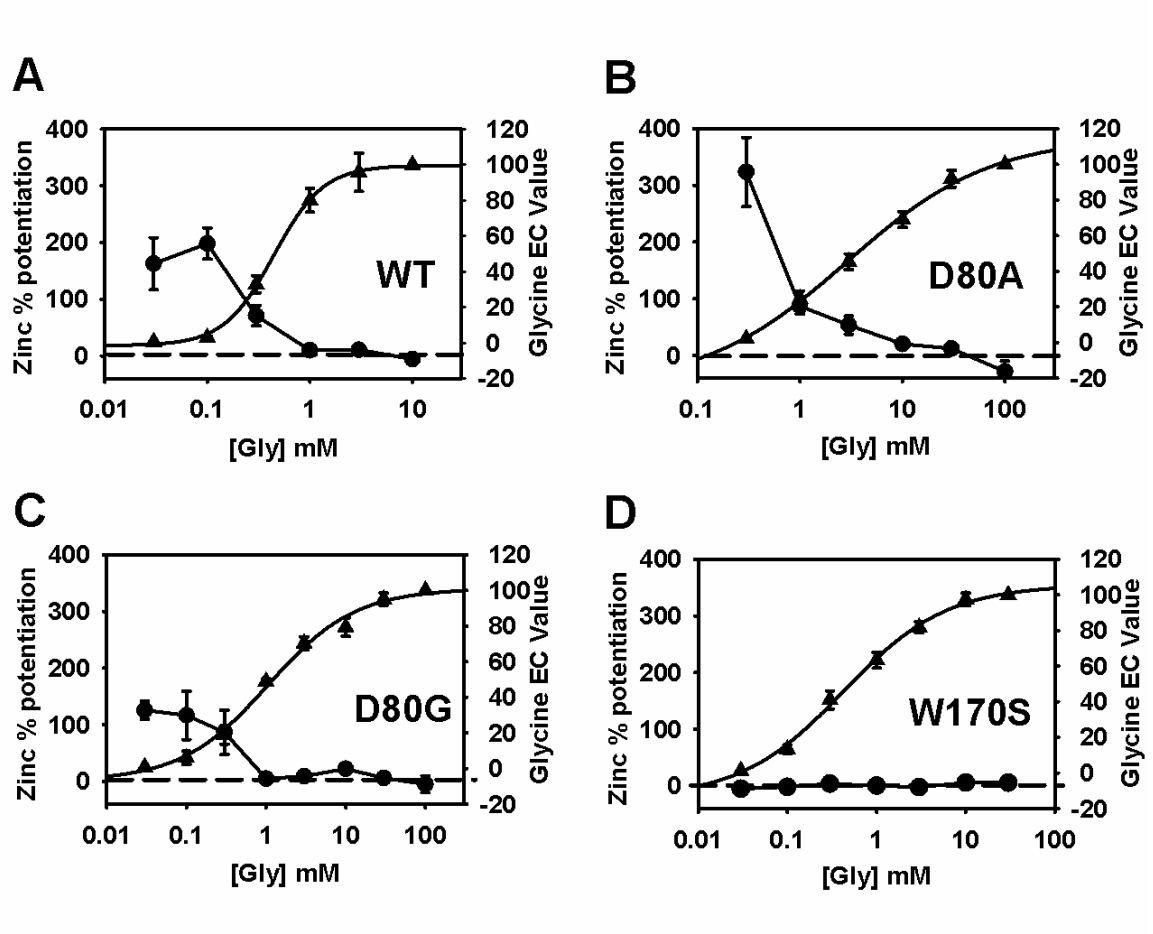

Figure 4.3: Zinc enhancement of wildtype and mutant receptors across agonist concentration response curves.

Zinc sensitivity of (A) WT, (B) D80A, (C) D80G, and (D) W170S glycine receptors was measured across different agonist concentrations. Glycine concentration response curves were determined (triangles) in the presence of 2.5 mM tricine and ordinate values are shown on the right axis. At each glycine concentration tested, the percent enhancement produced by 2.5 μM $ZnCl_2$ (in the presence of 2.5 mM tricine) was also determined (circles) and ordinate values are shown on the left axis. The dotted line corresponds to 0% zinc enhancement for reference. One-way ANOVAs showed that enhancement by $ZnCl_2$ decreased with increasing glycine concentration for WT [$F(5,30) = 15.435$, $p < 0.001$, n = 5-7], D80A [$F(5,21) = 24.153$, $p < 0.001$, n = 4-5], and D80G [$F(7,31) = 7.785$, $p < 0.001$, n = 4-7], but not W170S [$F(6,28) = 0.472$, $p = 0.823$, n = 3-6] GlyRs. Data are shown as the mean ± S.E.M. of the indicated number of oocytes obtained from at least two frogs.

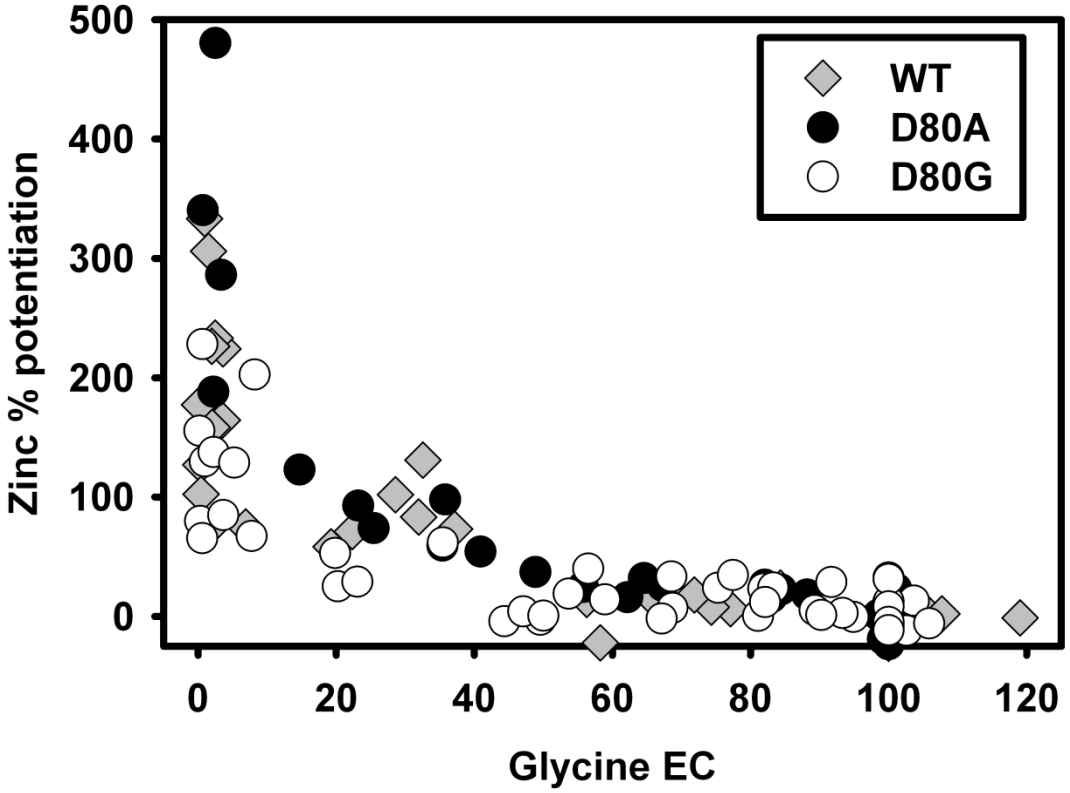

Figure 4.4: Zinc enhancement of WT and D80 mutant GlyRs as a function of glycine EC.

Zinc-enhancement of WT (grey diamonds), D80A (black circles), and D80G (white circles) GlyRs plotted against glycine EC. One-way ANOVAs revealed significant differences between genotypes at both $EC_{0\text{-}20}$ glycine [$F(2,26) = 5.812$, $p < 0.01$] and $EC_{20\text{-}40}$ glycine [$F(2,9) = 5.886$, $p < 0.05$]. Tukey multiple comparisons post-hoc tests showed that compared to WT receptors, zinc-enhancement of D80G receptors was significantly decreased at intermediate-low ($EC_{20\text{-}40}$) glycine (+38.70 ± 11.64% change for D80G compared to +92.07 ± 8.91% change for WT, $p < 0.01$), but not $EC_{0\text{-}20}$ glycine, suggesting a steeper decline in zinc mediated enhancement of D80G GlyRs with increasing agonist concentration.

4.3.2- Effects of zinc chelation on ethanol enhancement of wildtype and W170S GlyRs.

We next determined the effects of ethanol on W170S compared to wildtype GlyRs in the presence and absence of low nanomolar concentrations of zinc. To do this we measured ethanol enhancement of EC_{5-10} glycine currents for both wildtype and W170S receptors in normal MBS buffer (containing low nanomolar concentrations of contaminating zinc) and in buffer where contaminating zinc was chelated by tricine (**Fig. 4.5**). Consistent with McCracken et al. (2010), zinc-chelation by tricine significantly reduced enhancement of EC_{5-10} glycine currents by 50 and 200 mM ethanol in wildtype GlyRs. W170S GlyRs showed significantly decreased EtOH modulation compared to wildtype receptors in standard MBS and this was not further decreased by tricine. Additionally, there was no difference between the magnitudes of EtOH enhancement of wildtype and W170S GlyRs in the presence of tricine. Taken together, these data support the idea that the α1 W170S GlyR allows for the study of allosteric modulation of GlyRs in the absence of co-modulation by zinc.

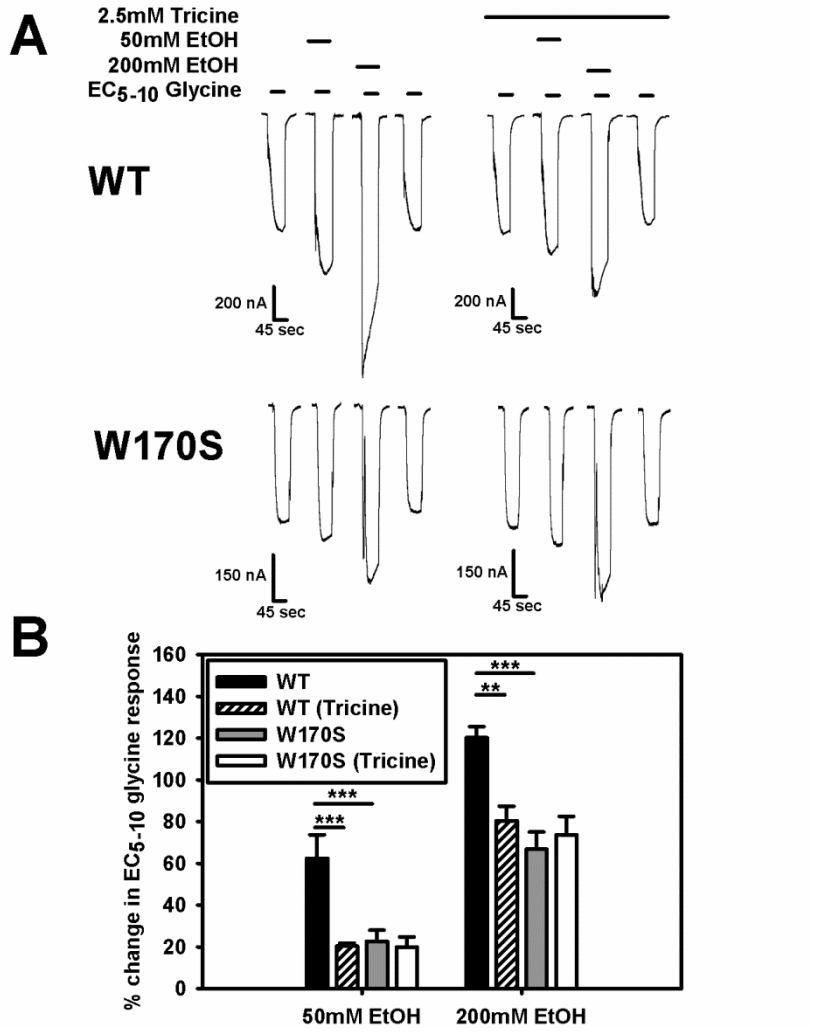

Figure 4.5: Effects of zinc chelation on ethanol enhancement of wildtype and W170S GlyRs

(A) Sample tracings showing the effects of zinc chelation on EtOH modulation of GlyRs activated by $EC_{5\text{-}10}$ glycine. The effects of 50 and 200 mM EtOH on $EC_{5\text{-}10}$ glycine currents were first determined in standard MBS, known to contain low nM concentrations of contaminating zinc, and then determined in MBS containing 2.5 mM of the zinc-chelator tricine. (B) Summary graph of the data presented in A. Zinc chelation significantly decreased the effects of 50 and 200 mM EtOH in WT GlyRs. Ethanol enhancement of W170S GlyRs in normal MBS was decreased compared to wildtype receptors and was not further decreased by the addition of tricine. There was also no difference in EtOH modulation between WT and W170S glycine receptors in the presence of tricine, indicating the W170S mutation allows for accurate depiction of allosteric modulation in the absence of co-modulation by zinc. Data are shown as the mean ± S.E.M. of 4-7 oocytes obtained from at least two frogs. Two-way ANOVA followed by Tukey's post-hoc tests: **, $P < 0.01$. ***, $P < 0.001$.

4.4- Discussion

Apart from the ability of nanomolar concentrations of zinc to allosterically enhance GlyR function, low concentrations of zinc also interact with other allosteric GlyR modulators, such as ethanol, yielding effects greater than those produced by zinc or modulator applied alone (Cornelison et al., 2016; McCracken et al., 2010, 2013a,b). We generally account for this phenomenon via the removal of "free zinc" from our buffers by the addition of chelating agents such as tricine. However, these data did not discount the possibility that the addition of chelating agents such as tricine might have effects on receptor activation or allosteric modulation by means distinct from their abilities to chelate zinc. Testing for the effects of allosteric modulators on GlyR mutants resistant to the enhancing properties of zinc would alleviate this concern. We first attempted to use homomeric α1 D80A and D80G mutant GlyRs that were previously reported to be insensitive to the enhancing effects of zinc (Hirzel et al., 2006; Laube et al., 2000; Lynch et al., 1998; McCracken et al., 2013a). However, our data revealed that these two mutants are not truly insensitive to enhancing concentrations of zinc (**Fig. 4.1**). We then screened the α1 W170S GlyR and found that both tricine and 100nM $ZnCl_2$ had negligible effects on W170S GlyR currents (**Fig. 4.1**), confirming this mutant's reported lack of zinc enhancement and showing that tricine does not act as a modulator of GlyRs beyond its ability to chelate zinc. This result also served as a positive control, markedly contrasting with the findings made using the two D80 mutants.

The discrepancies between our data and previously published reports on the zinc-insensitive nature of α1 GlyR D80 mutants prompted a more thorough characterization of their sensitivities to enhancing concentrations of zinc. We first tested for the effects of a variety of GlyR-enhancing zinc concentrations on submaximal glycine currents in WT

and mutant receptors (**Fig. 4.2**). While WT and D80A receptors showed similar zinc responses across the concentrations tested, D80G receptors had a much shallower concentration-response curve with significantly lower peak enhancement. We have previously determined that our electrophysiological buffers (MBS) contain approximately 45 nM concentrations of contaminating zinc (Cornelison and Mihic, 2014), a concentration that is similar to that found tonically in CSF (Frederickson et al., 2006b). However, other studies have reported up to approximately 200-800 nM contaminating concentrations of zinc in various electrophysiological buffers, suggesting that this could be a significant confound in the many published studies in the GlyR field (Thio and Zhang, 2006; Wilkins and Smart, 2002; Zheng et al., 1998). As contaminating zinc has not generally been taken into consideration, the higher levels of zinc contamination in buffers used for previous studies of D80G GlyRs may have masked the effects of the experimentally added zinc in mutant receptors while still noticeably enhancing WT receptors. However this would not explain why we saw at least as great an effect of zinc in D80A as in WT α1 GlyRs (**Fig. 4.2**). We next investigated GlyR sensitivity to zinc on currents generated by a variety of agonist concentrations. Allosteric modulators of ligand gated ion channels typically show the greatest effects at low agonist concentrations, with a decrease in percent enhancement occurring with increasing agonist concentration (Farley and Mihic, 2015). Since previous studies of α1 D80 mutant GlyRs typically assayed zinc-sensitivity using higher glycine concentrations than reported here ($\geq EC_{25}$), we hypothesized that perhaps D80 mutants might have a steeper decline in zinc-enhancement with increasing agonist concentrations than WT receptors, thereby appearing insensitive to zinc at currents generated by higher effective concentrations of glycine. We found that D80A, D80G, and WT receptors all showed a decrease in zinc enhancement with increasing agonist concentration (**Fig. 4.3**). However, D80G receptors

had significantly reduced zinc potentiation compared to WT receptors when activated by glycine concentrations ranging from EC_{20-40} but not EC_{0-20} (**Fig. 4.4**). These data suggest that a steeper decline in zinc-enhancement with increasing agonist concentration, coupled with the possible presence of higher levels of contaminating zinc than in our buffers, may account for previous reports of abolished zinc enhancement in D80G GlyRs. Once again, this would not explain why D80A GlyRs show such marked zinc enhancement in our hands.

The exact GlyR residues that are involved in the high affinity binding of zinc remain unclear. Our data suggests that D80 is not part of a high affinity zinc-binding site, as robust enhancement of receptor function by zinc is seen in both D80A and D80G GlyRs expressed in *Xenopus laevis* oocytes. Other studies of D80 GlyR mutants are also contradictory as to whether D80 is a zinc binding residue. Lynch et al. (1998) found that 1 μM zinc significantly increased the apparent affinities of both glycine and taurine in [^{3}H]-strychnine binding assays of D80A mutant receptors, consistent with a mechanism of positive allosteric modulation. Functionally, however, they found that glycine-mediated currents in D80A GlyRs were insensitive to enhancement by zinc while taurine-mediated currents were still enhanced. They concluded that instead of acting as a zinc-binding site, D80 may instead be involved in allosterically linking zinc binding to channel gating. While our results could possibly account for the apparent lack of zinc enhancement in previous reports of D80G GlyRs, we were unable to do the same for the D80A receptor. One speculative possibility is that D80A mutations differentially affect zinc enhancement of receptors expressed in different cell types as previous studies characterizing D80A receptors were conducted in mammalian cells while we utilized *Xenopus laevis* oocytes.

Another putative high-affinity zinc binding site associated with GlyR enhancement involves residues E192 and D194 of the α1 subunit (Miller et al., 2005). W170 is in very close proximity to these residues and mutation of W170 to serine is thought to disrupt this binding pocket, thereby preventing enhancement by zinc (Zhou et al., 2013). Since we were able to successfully confirm the zinc-insensitive nature of this mutant, we investigated its utility as a model for studying GlyR modulation in the absence of co-modulation by zinc. We did this by testing the effects of ethanol and tricine on W170S GlyRs in comparison with the effects of tricine on ethanol modulation of WT receptors (**Fig. 4.5**). W170S receptors had a significantly decreased response to ethanol compared to WT receptors in the presence of contaminating (low nM) concentrations of zinc and this ethanol effect was not further decreased by tricine. Additionally, the degree of ethanol enhancement of W170S GlyRs was similar to what was seen for WT receptors in the presence of tricine. This suggests that tricine has no effect on ethanol modulation of GlyRs apart from its ability to chelate zinc and demonstrates that the W170S receptor serves as an appropriate model to investigate modulator function in the absence of co-modulation by zinc.

Chapter 5: Insights into zinc's interactions with allosteric modulators of the glycine receptor

5.1- Introduction

As discussed previously, it is now evident that contaminating levels of zinc are capable of affecting the modulation of GlyR function by other compounds (See chapter 3; Cornelison et al., 2016; McCracken et al., 2010, 2013a,b). Specifically, it appears that the presence of low nanomolar concentrations of zinc act synergistically with positive allosteric modulators, such as ethanol, to enhance receptor function. However, previous studies characterizing allosteric modulation of the GlyR have seldom accounted for the presence of contaminating zinc, therefore limiting our knowledge as to the extent of this phenomenon. In the previous chapter we validated the utility of using the zinc-enhancement insensitive α1 W170S GlyR to study GlyR modulation in the absence of co-modulation by zinc. In this chapter we utilize this mutant receptor to answer several open questions about zinc's ability to interact with other modulators of GlyR function.

5.2- Materials and Methods

5.2.1- Two-electrode voltage-clamp electrophysiology

Oocyte isolation, injection, and electrophysiological recordings were performed as described in chapter 2. Glycine and modulators were diluted in, and all electrophysiological recordings were performed in, modified Barth's saline (MBS) buffer or MBS + 10 μM $ZnCl_2$. For all experiments, modulators were pre-incubated for 30 sec prior to co-application with glycine. When maximally-effective concentrations of glycine were used, applications lasted for 10 sec and were followed by a 15 min washout to allow for full receptor re-sensitization. When EC_{5-10} concentrations of glycine were

used, applications lasted 45 sec followed by a 5 minute washout. For all experiments using EC_{5-10} glycine, maximally-effective glycine responses were first determined and used to find the EC_{5-10} glycine. This process was repeated for each condition tested (i.e. MBS and MBS + 10 μM $ZnCl_2$). Every EC_{5-10} glycine + modulator co-application was flanked by an EC_{5-10} glycine control for comparison. Maximally-effective glycine responses were checked again at the end of every experiment to account for drift in glycine responses over time. Applications in which control EC_{5-10} glycine responses drifted above EC_{10} or below EC_5 were not used. Loss of volatile compounds through tubing and evaporation from bath was previously measured (Beckstead et al., 2000; Mihic et al., 1994; Yamakura et al., 1999). All concentrations reported are the bath concentrations to which the oocytes were exposed. All data were collected from oocytes obtained from at least two different frogs.

5.2.2- Data analysis

Peak currents were measured and used in data analysis. For each oocyte, currents observed in the presence of glycine plus modulators were compared with currents generated by glycine alone and expressed as the mean ± S.E.M of the percent change in glycine-mediated current. Significant differences were determined using the Student's t-test, two-way ANOVA, and Tukey post-hoc tests, as indicated. All statistical testing was performed using SigmaPlot 11.0 (Systat Software, San Jose, CA).

5.3- Results

5.3.1- Peptide modulation of wildtype and W170S GlyRs

First, we verified the ability of D7.2-123-B to enhance GlyR function in a zinc-independent manner. This peptide was identified from a phage display screen targeting the α1β GlyR (see chapter 3). However, we were unable to successfully express functional α1(W170S):β receptors and therefore compared peptide enhancement of homomeric α1 wildtype and W170S receptors (**Fig 5.1)**. Co-application of 30 μM D7.2-123-B with EC_{5-10} glycine potentiated homomeric wildtype receptor currents by 132.0%, similar to what was seen in α1β receptors **(Fig 3.2)**, and potentiated W170S homomeric receptors by 43.52%.

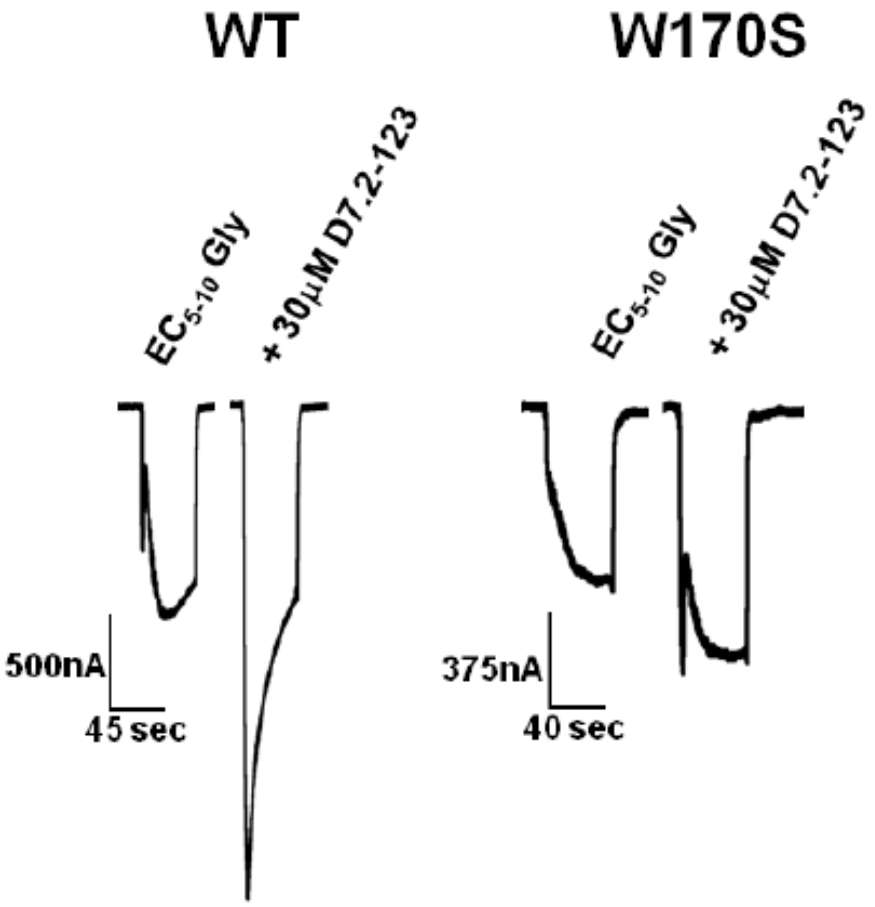

Figure 5.1: D7.2-123-B modulation of α1 homomeric wtildypte and W170S GlyRs

Peptide D7.2-123-B, tested at a concentration of 30 μM, conferred 132.0% enhancement in wildtype α1 GlyRs and 43.52% potentiation of EC_{5-10} glycine currents in α1 W170S mutant receptors.

5.3.2- Volatile anesthetic and inhalant sensitivity of wildtype and W170S GlyRs under low nanomolar zinc conditions.

We next used W170S GlyRs to probe for the involvement of low nanomolar concentrations of zinc on GlyR enhancement by other drugs of abuse. The enhancement of EC_{5-10} glycine currents in wildtype and W170S GlyRs produced by several volatile anesthetics and inhaled drugs of abuse was determined in standard MBS (containing low nanomolar zinc). There were no differences in GlyR enhancement between WT and W170S receptors by approximately minimum alveolar concentrations (MAC) of the volatile anesthetics isoflurane, chloroform, or halothane (**Fig. 5.26A**). The MAC represents an anesthetic's ED_{50} in humans. On the other hand, the inhalants 1,1,1-trichloroethane (TCE), toluene, and trichloroethylene (TCY) showed a significantly decreased ability to affect GlyR function in W170S compared to WT receptors (**Fig. 5.2B**), suggesting an interaction between zinc and inhalants to enhance GlyR function.

5.3.3- Allosteric modulator sensitivity in wildtype and W170S GlyRs under low micromolar zinc conditions.

Previous studies investigating the interaction between zinc and other allosteric modulators at the GlyR have focused on zinc concentrations ≤ 1 μM, in which only the high affinity GlyR-enhancing zinc binding site would be occupied. However, synaptically released zinc may result in higher local concentrations which may occupy both the high affinity enhancing and low affinity inhibitory zinc binding sites. We therefore measured the effects of exogenously-added 10 μM $ZnCl_2$ on the modulation of EC_{5-10} glycine currents in WT and W170S GlyRs by ethanol, (**Fig. 5.3**) TCE, and halothane (**Fig. 5.4**). **Figure 5.3A** shows sample tracings of ethanol's effects on EC_{5-10} glycine currents from wildtype and W170S GlyRs in normal MBS and MBS + 10 μM $ZnCl_2$. Enhancement of EC_{5-10} glycine currents by 50 and 200 mM ethanol were

significantly decreased in WT GlyRs when 10 μM $ZnCl_2$ was added to the buffer. However, ethanol modulation of W170S GlyRs compared to WT receptors was already significantly decreased in standard MBS and was not further affected by the addition of zinc. Additionally, there were no significant differences between ethanol modulation of WT and W170S GlyRs in the presence of 10 μM $ZnCl_2$. A similar pattern was seen for TCE, in which the addition of 10 μM $ZnCl_2$ significantly decreased TCE enhancement for WT but not W170S GlyRs, with no significant differences between WT and W170S receptors in MBS + 10 μM $ZnCl_2$. (**Fig. 5.4**). There were no significant differences in halothane modulation of WT and W170S GlyRs, regardless of the concentration of zinc present (**Fig. 5.4**).

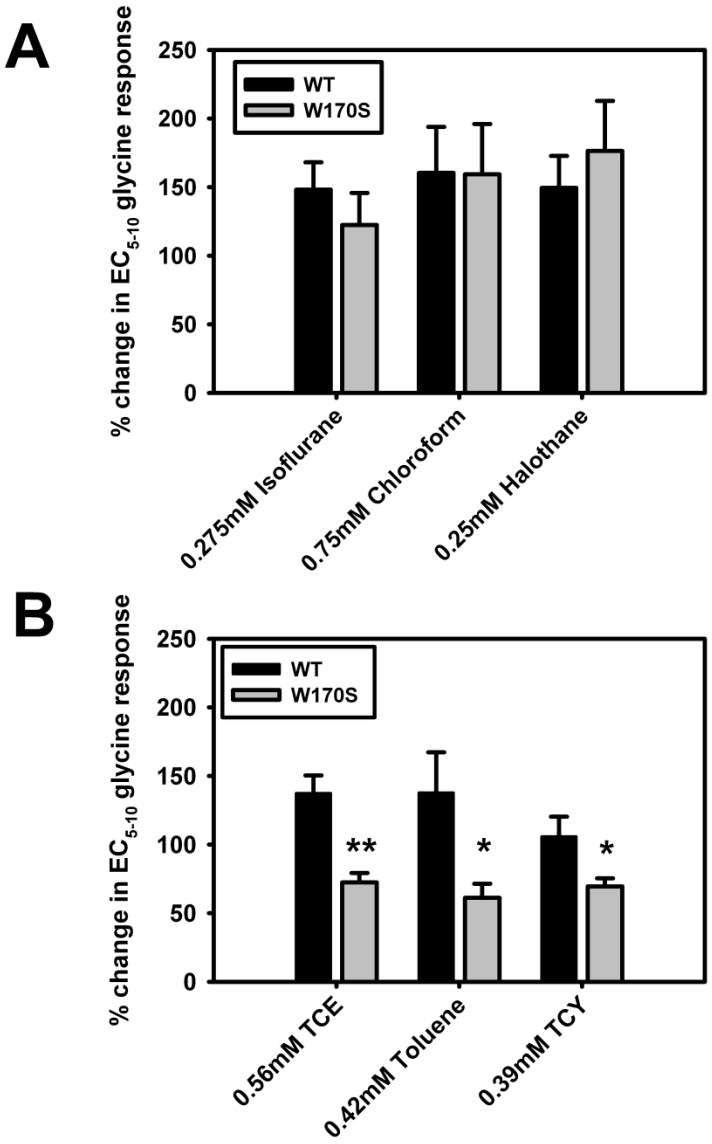

Figure 5.2: Co-modulation by low nanomolar zinc affects allosteric modulation of glycine receptors by inhalants but not volatile anesthetics.

(A) Summary graph of the effects of approximately minimum alveolar concentrations of three volatile anesthetics on WT and W170S glycine receptors activated by $EC_{5\text{-}10}$ glycine. No significant differences were seen. Data shown as the mean ± S.E.M. of 4-5 oocytes obtained from at least two frogs. (B) Summary graph of the effects of inhalants on WT and W170S glycine receptors activated by $EC_{5\text{-}10}$ glycine. Data are shown as the mean ± S.E.M. of 5 oocytes obtained from at least two frogs. Student's t-test *, $P < 0.05$. **, $P < 0.01$.

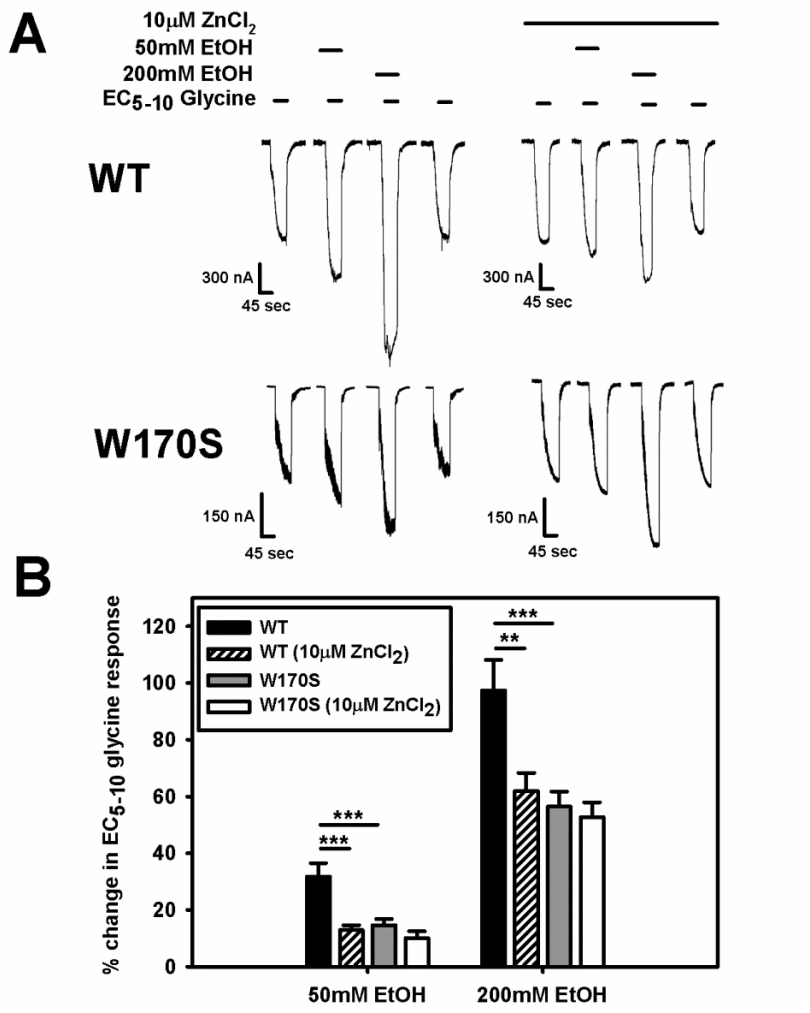

Figure 5.3: Higher concentrations of zinc, which may be found after synaptic release, inhibit EtOH enhancement of α1 WT but not W170S GlyRs.

(A) Sample tracings showing the effect of 10 μM added $ZnCl_2$ on EtOH modulation of α1 WT and W170S GlyRs activated by $EC_{5\text{-}10}$ glycine. The effects of 50 and 200 mM EtOH on $EC_{5\text{-}10}$ glycine currents were first determined in standard MBS buffer, known to contain low nM concentrations of contaminating zinc, and then determined in MBS containing 10 μM $ZnCl_2$. (B) Summary graph of the data presented in A. The addition of 10 μM $ZnCl_2$ to the buffer significantly decreased the effects of 50 and 200 mM EtOH in WT GlyRs. Ethanol enhancement of W170S GlyRs in normal MBS was decreased compared to wildtype receptors and was not further decreased by added $ZnCl_2$. There was also no difference in EtOH modulation between WT and W170S GlyRs in the presence of added $ZnCl_2$. Data are shown as the mean ± S.E.M. of 5-6 oocytes obtained from at least two frogs. Two-way ANOVA followed by Tukey's post-hoc tests: **, $P < 0.01$. ***, $P < 0.001$.

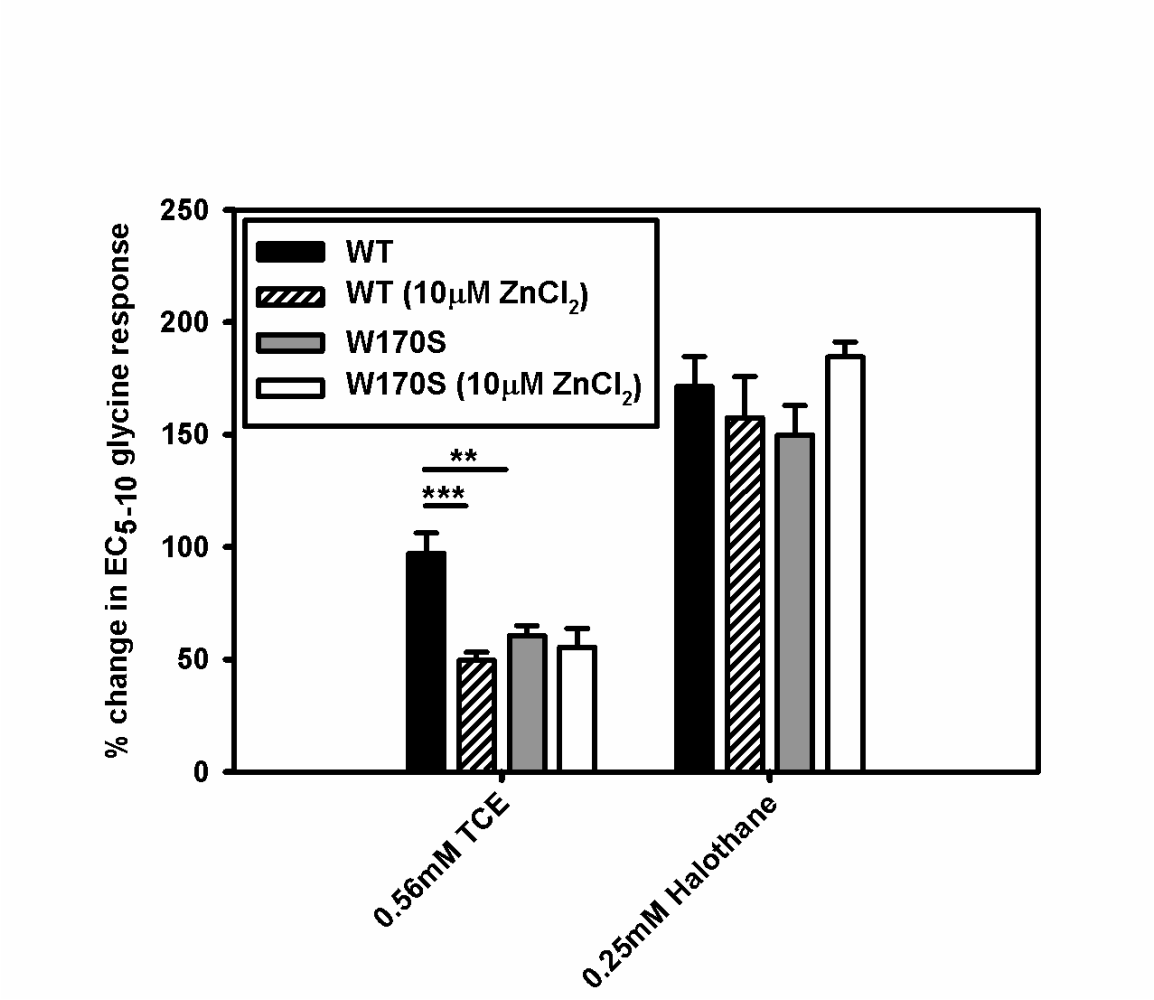

Figure 5.4: Higher concentrations of zinc inhibit TCE but not halothane enhancement of α1 WT but not W170S GlyRs.

The addition of 10 μM $ZnCl_2$ to the buffer significantly decreased the effects of 0.56 mM TCE in WT GlyRs. TCE enhancement of W170S GlyRs in normal MBS was decreased compared to wildtype receptors and was not further decreased by the addition of $ZnCl_2$. There was also no difference in TCE modulation between WT and W170S receptors in the presence of added $ZnCl_2$. There were no significant differences in halothane modulation of WT and W170S GlyRs in the presence or absence of added $ZnCl_2$. Data are shown as the mean ± S.E.M. of 4 oocytes obtained from at least two frogs. Two-way ANOVA followed by Tukey's post-hoc tests: **, $P < 0.01$. ***, $P < 0.001$.

5.4- Discussion

In chapter 4 we investigated the utility of α1 W170S mutant GlyRs for the study of GlyR modulation in the absence of co-modulation by enhancing concentration of zinc. Nanomolar concentrations of zinc have been shown to synergistically enhance ethanol modulation of GlyR function (McCracken et al., 2010, 2013a,b). Further, it appears that modulation of GlyR function by recently discovered peptides is zinc-dependent, as the chelation of contaminating zinc abolished their ability to affect channel function (See chapter 3; Cornelison et al., 2016). Due to the ubiquitous nature of zinc in the CNS, it is important to account for its ability to affect other modulators of GlyR function in order to have a better understanding of how these compounds work *in vivo*. We therefore utilized the W170S mutant GlyR to investigate a variety of open questions involving zinc's interactions with other modulators at the GlyR.

We first tested for the ability of D7.2-123-B to enhance W170S GlyR function. D7.2-123-B was one of the most potent peptides identified in chapter 3, but also contained a significant amount of contaminating zinc that could possibly account for its enhancing effects. ICP-MS measurements revealed the presence of over 100 nM contaminating zinc at the peptide concentrations tested electrophysiologically. However, ICP-MS analysis only measures total zinc, and not the concentration of free zinc that would be able to act on the receptor. Chelation of contaminating zinc with tricine significantly reduced D7.2-123-B potentiation of GlyR function, suggesting that either contaminating zinc is responsible for some of the observed peptide effects or that peptide action is zinc-dependent, similar to ethanol. The fact that tricine also inhibited modulation produced by other peptides, containing minimal concentrations of contaminating zinc, suggests zinc-dependence of peptide action. However, it is possible

that the presence of such a high concentration of tricine (2.5 mM) could affect peptide modulation in a manner independent of zinc chelation, such as through alteration of the conformational state of the peptide. We therefore tested for the ability of D7.2-123-B to enhance α1 WT and W170S GlyR function. Unfortunately, we were unable to express functional heteromeric α1(W170S):β receptors. However, D7.2-123-B enhanced WT α1 homomeric and α1β heteromeric receptors to a similar degree, suggesting that it should be able to enhance homomeric α1 W170S GlyR function as well (**Fig. 5.1**). We saw that D7.2-123-B enhancement was significantly decreased in W170S GlyRs compared to wildtype receptors, similar to the decrease of peptide enhancement seen in α1β receptors in the presence of tricine (**Fig. 5.1**). This suggests tricine inhibition of peptide modulation is mediated through the chelation of zinc and not some effect of tricine on the peptides themselves.

We next used the W170S receptor to probe for zinc's ability to interact with other GlyR modulators, finding significantly reduced enhancement by inhalants in W170S GlyRs, suggesting that these modulators also interact with zinc to synergistically enhance receptor function (**Fig. 5.2**). However, this was not true for GlyR enhancement by volatile anesthetics, which had similar effects in WT and W170S receptors, suggesting that there are some differences in how anesthetics enhance GlyR function, compared to ethanol and inhaled drugs of abuse.

Previous studies investigating zinc's interactions with other GlyR modulators examined zinc concentrations ≤ 1 μM. However, much higher concentrations of zinc may be present after synaptic release. We therefore investigated the effects of 10 μM $ZnCl_2$ on ethanol modulation (**Fig. 5.3**). Interestingly, we saw that enhancement by ethanol was significantly decreased in WT receptors in the presence of 10 μM zinc compared to standard MBS, where only ~45 nM zinc would be present. One possible

explanation is that occupation of the inhibitory zinc-binding sites inhibits the ability for ethanol to modulate the channel. However, when we repeated this experiment with W170S receptors, which only exhibit zinc inhibition, 10 μM zinc had no effect on ethanol modulation. This experiment suggests that saturation of the zinc-enhancing site, not occupation of the inhibitory site, results in the decrease in ethanol's enhancement seen in WT receptors. While this seems counter-intuitive with the findings that lower concentrations of zinc act synergistically with ethanol to enhance GlyR function, these data fit nicely with a mechanism by which ethanol, in addition to modulating the channel on its own, increases the affinity for zinc at the GlyR-enhancing site. The lower degree of ethanol enhancement seen in the presence of tricine reflects the ability of ethanol itself to enhance GlyR function. In the presence of lower concentrations of zinc, ethanol's enhancement appears larger because in addition to its own modulation of channel function, it increases the affinity of zinc binding to the GlyR-enhancing site, thereby increasing enhancement by zinc as well. However, in the presence of higher concentrations of zinc, the GlyR-enhancing zinc site is already saturated and once again the effects that are seen are those produced by ethanol alone. The similar results observed with TCE suggest that inhalants interact with zinc by the same mechanism. However, halothane enhancement was unaffected by zinc concentration in WT and W170S receptors, providing further support that volatile anesthetic modulation of GlyRs is not affected by the presence of zinc and that not all of these allosteric modulators act by the same exact mechanisms.

Chapter 6: General discussion, conclusions, and future directions

6.1- Overview

Pharmacological enhancement of GlyR function has been proposed as a promising strategy for the treatment of both alcoholism and chronic inflammatory pain (Li et al., 2012; Molander et al., 2005; Molander and Soderpalm, 2005a; Xiong et al., 2011, 2012). However, the lack in specificity of currently known GlyR modulators has hindered the development of promising candidate molecules for GlyR-targeted therapeutic applications. Indeed, most positive allosteric modulators of GlyR function, such as ethanol, cannabinoids, and zinc, are capable of affecting a wide variety of other molecular targets. Further, the magnitude by which some modulators affect GlyR function is dependent upon the local concentration of zinc present at the receptor. Since zinc is ubiquitous in the CNS, it is critical to account for any possible interaction between zinc and other GlyR modulators in order to have a clear understanding of how these molecules behave *in vivo.* The research presented in this dissertation expands on a recently-developed method to identify specific modulators of GlyR function, highlights the importance of accounting for zinc when characterizing novel GlyR modulators, and describes a possible mechanism by which modulators interact with zinc to enhance receptor function.

6.2- Phage display for the discovery of novel modulators of GlyR function

Tipps et al. (2010) reported the first use of phage display technology for the identification of allosteric modulators of a ligand-gated ion channel. Utilizing

commercially available phage display libraries and a HEK cell based panning procedure, they were able to identify peptides capable of selectively enhancing GlyR function at low μM concentrations. In chapter 3, we expanded on this procedure in an attempt to identify peptides capable of selectivity modulating specific GlyR subtypes. GlyR subunits can combine to form six possible subtypes: α1, α2, and α3 homomeric receptors or α1β, α2β, and α3β heteromeric receptors (Lynch et al., 2009). While α3-containing receptors are thought to predominate in GlyR-mediated analgesia (Xiong et al., 2011, 2012), it is not entirely clear which subtypes are responsible for the behavioral and dopamine-modulating effects of alcohol. Therefore, subtype selective GlyR modulators could serve as valuable pharmacological tools in determining which GlyR subunits are important for various physiological functions, such as responses to ethanol.

Recent behavioral studies of ethanol's effects on GlyR mutant mice suggest that α1-containing GlyRs regulate ethanol-induced motor activity and sedation while α2-containing receptors seem the most important in mediating ethanol preference and consumption (Aguayo et al., 2014; Blednov et al., 2015; Findlay et al., 2002). Specifically, α2 GlyR knockout mice showed decreased voluntary ethanol consumption as assayed by a 24 hour two-bottle choice test (Blednov et al., 2015). While this may seem counterintuitive to the findings that increased accumbal GlyR function decreases ethanol consumption and preference in rats (Li et al., 2012; Molander et al., 2005), it makes sense when considered in the context that GlyRs control ethanol-induced dopamine release and therefore gate the reward signal for consuming alcohol. For example, if ethanol-induced dopamine release is significantly inhibited in α2-knockout mice, than they will not find ethanol as rewarding and therefore drink less than wildtype controls, as was seen by Blednov et al. (2015). In support of the role of the α2 subunit in ethanol consumption, recent reports on GlyR transcript levels in rats identified the α2

subunit as the most highly expressed α subunit in the forebrain, including regions important in the rewarding properties of ethanol such as the nAc (Delaney et al., 2010; Jonsson et al., 2009, 2012).

As we obtained peptides with selectivity for α1β and α3β compared to α2β receptors, future phage display procedures targeting the α2 subunit are warranted. If novel α2-selective GlyR-enhancing peptides were capable of reducing voluntary ethanol consumption, while the α1- and α3-selective peptides described here were not, this would validate the α2 GlyR subunit as the primary molecular target mediating ethanol intake. However, due to the effectiveness of the presently-reported peptides to enhance α3β GlyR function, they may still serve as useful leads in the development of a novel medication for the treatment of chronic inflammatory pain. Specifically, the deduced consensus sequences described in **table 3.2** could be utilized in directing the production of novel phage display libraries in which each clone would display a variant of these sequences. This would allow for the possible identification of higher potency peptides that may be able to enhance GlyR function in a clinically relevant range; i.e. low nanomolar concentrations.

6.3- Zinc's interactions with other allosteric modulators of GlyR function.

Zinc is one of the most prevalent nutritionally-essential elements in the human body and is the most abundant trace metal in the brain (Watt 2013; Tapiero 2003). Zinc is critical to the structure and function of a large number of macromolecules and is essential for over 300 enzymatic reactions (Tapiero, 2003). Long known as a potent modulator of GlyR function, zinc is interesting in that it serves as a biphasic modulator of

the GlyR, enhancing GlyR activity at concentrations in the low nM to 10 μM range and inhibiting GlyR activity at higher concentrations (Laube et al., 1995). Further, zinc influences the degree by which ethanol enhances GlyR function, increasing ethanol potentiation when nanomolar concentrations of zinc are present (McCracken et al., 2010, 2013a,b). The ubiquitous nature of zinc, found both physiologically in the CNS and as contaminant in labware and reagents, makes it critical to account for the effects of zinc in studies of GlyR function and modulation.

In chapter 3 we tested the hypothesis that peptide modulation of GlyR function was zinc-dependent, similar to modulation by ethanol. The addition of tricine to our electrophysiological buffer significantly inhibited the ability of peptide modulators to affect receptor function, suggestive of zinc dependence. However, these data did not discount the possibility that the addition of tricine might have effects on receptor activation or allosteric modulation apart from their abilities to chelate zinc. For example, tricine could act itself as an allosteric modulator of GlyR function or it could somehow inhibit the ability of peptides to bind the channel, or conceivably even bind to the peptides themselves. In order to alleviate this concern we investigated the utility of mutant GlyRs previously reported as insensitive to enhancement by zinc for the study of GlyR modulation in the absence of any effects from contaminating zinc (Chapter 4). While we were unable to confirm the previously-reported results of abolished zinc enhancement in α1 D80A and D80G GlyRs, we were able to confirm this for the α1 W170S GlyR. Using this mutant receptor, we confirmed that tricine itself does not appear to modulate GlyR function. Further, ethanol enhancement of W170S receptors was similar to that of WT receptors when zinc was chelated with tricine, suggesting this mutant receptor can serve as an adequate model for studying GlyR modulation in the absence of co-modulation by contaminating zinc.

In chapter 5 we utilized the α1 W170S GlyR to address several open questions involving zinc. First, we confirmed the zinc-dependence of peptide D7.2-123-B enhancement of GlyR function. This suggests tricine's effect on peptide modulation was due purely to the chelation of zinc and not any effect of tricine on the peptides themselves. However, as every peptide behaves differently based on the chemical properties of its unique amino acid sequence, this conclusion should not be generalized to all peptides. Secondly, we used the W170S receptor to screen for zinc-dependence in the action of volatile anesthetics and inhaled drugs of abuse at the GlyR. Interestingly, we found that GlyR modulation by inhalants but not volatile anesthetics appears zinc dependent. This is surprising as ethanol, inhalants, and volatile anesthetics all share an overlapping binding site (Beckstead et al., 2000; Mihic et al., 1997), and suggests that these modulators must interact with this binding pocket in different ways to enhance GlyR function. However, there are no clear differences in the structures of volatile anesthetics compared to ethanol or inhalants that could account for their apparent difference in mechanism in relation to zinc (see page xviii). Molecular dynamics simulations of how the positions of these modulators fluctuate within the binding pocket might identify key contacts necessary for the interaction between modulators and zinc. Finally, testing for the effects of higher concentrations of zinc on modulation of α1 WT compared to W170S GlyRs allowed us to deduce a mechanism by which molecules such as ethanol and inhalants interact with zinc to increase channel function. Specifically, these compounds, besides being able to positively modulate receptor function on their own, also appear to increase the affinity of zinc to its GlyR-enhancing binding site, thereby increasing zinc enhancement of the channel as well. However, as these experiments were not performed with peptides, it is unclear if their zinc-dependence is mediated through the same mechanism. An alternative mechanism for zinc-dependence

of peptide action is that zinc could be important in maintaining peptides in their GlyR-binding conformation.

The local concentration of free zinc that is available to act on GlyRs in the CNS is unclear. Tonic concentrations of zinc in human CSF are in the low nanomolar range, similar to what is found in our electrophysiological buffers (Frederickson et al., 2006b). However, zinc is also released synaptically, resulting in local concentrations of zinc that have been reported to range from 1-100 μM (Frederickson et al., 2006a; Qian and Noebels, 2005; Vogt et al., 2000; Zhang et al., 2016). Further, a recent report using artificial synapses created from cultured rat embryonic glycinergic neurons suggested that they may be capable of synaptically releasing zinc (Zhang et al., 2016). Since modulators such as ethanol have the highest level of enhancement at nanomolar concentrations of zinc with decreased enhancement seen at μM concentrations (See chapter 5; McCracken et al., 2013a,b), future studies should be directed at determining the extent of synaptically-released zinc's involvement in endogenous modulation of the GlyR and how this influences channel modulation by potential therapeutics or drugs of abuse.

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Vita

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